DESIGN, SYNTHESIS AND EVALUATION OF CYSTEINE PROTEASE INHIBITORS

A Dissertation
Presented to
The Academic Faculty

by

Asli Ovat

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in the
School of Chemistry and Biochemistry

Georgia Institute of Technology
May 2009

DESIGN, SYNTHESIS AND EVALUATION OF CYSTEINE PROTEASE INHIBITORS

Approved by:

Dr. James C. Powers, Advisor
School of Chemistry and Biochemistry
Georgia Institute of Technology

Dr. Christoph J. Fahrni
School of Chemistry and Biochemistry
Georgia Institute of Technology

Dr. Sheldon W. May
School of Chemistry and Biochemistry
Georgia Institute of Technology

Dr. Jonathan D. Glass
School of Medicine
Emory University

Dr. Nicholas V. Hud
School of Chemistry and Biochemistry
Georgia Institute of Technology

Date Approved: April 1, 2009

To my mother, Fatma Ovat

Canim Annem, Fatma Ovat'a

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my advisor, James C. Powers. It has been an honor and pleasure to work under your guidance and I will miss our talks about life and movies.

I would like to thank my committee members; Dr. May, Dr. Doyle, Dr. Fahrni and Dr. Glass for taking their time to read my thesis, listen to my research and for their guidance.

I would also like to thank the past and present members of Powers group; Dr. Zhao Zhao Li, Dr. Amy Campbell, Benison Forfeke and Temam Juhar.

I would like to thank all my Turkish friends at Georgia Tech, known as "Turkish Mafia". Alpay Kimyonok, Ozgul Persil, Mehmet Cetinkol, Gozde Guler, Burak Boz, Gungor Ozer and Selma Bakbak; I will always cherish your friendship. And special thanks go to my support team in Boston. Gulbenk Anarat, Diren Pamuk, Deniz Yuksel and Serkan Yurt, I have always felt your support even we were far away.

My dear parents, Necati and Fatma Ovat, I am grateful for your endless support and encouragement. My dear mom, I know that I can not thank you enough but you should know that you mean the world to me and none of this could be done without you. Thank you for being such a great mom. My little sisters, Hande and Nihan Ovat, you are everything for me. No matter how far we were and no matter how tough things were, I knew that you were just a phone call away to make me smile. I love you very much.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

AA	amino acid residue
AAsn	aza-asparagine
Abu	4-aminobutyric acid
Ac	acetyl
AE	asparaginyl endopeptidase
AHph	aza-homophenylalanine
Ala	alanine
AMC	7-amino-4-methylcoumarin
Arg	arginine
Asn	asparagine
Asp	aspartic acid
Boc	*tert*-butoxycarbonyl
Boc-Piz	*tert*-butoxycarbonylpiperazine-CO
Brij	polyoxyethylenelaurylether
Bzl	benzyl, CH_2Ph
Cbz	benzyloxycarbonyl
Cbz-Piz	benzyloxycarbonylpiperazine-CO
$CDCl_3$	deuterated chloroform
CH_2Cl_2	methylene chloride
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
Cys	cysteine
DCC	1,3-dicyclohexylcarbodiimide
DMAP	4-dimethylaminopyridine

DMF	*N,N*-dimethylformamide
DMSO	dimethylsulfoxide
DMSO-d_6	dimthylsulfoxide-d_6 deuterated
DTT	dithiothreitol
E-64	L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane
EDC	1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycoltetraacetic acid
EP	epoxide
ESI-MS	electrospray ionization mass spectrometry
Et_3N	triethylamine
Et_2O	diethyl ether
EtOAc	ethyl acetate
EtOH	ethanol
FAB	fast atom bombardment
Gln	glutamine
Glu	glutamic acid
Gly	glycine
HCl	hydrochloric acid
His	histidine
HOBt	*N*-hydroxybenzotriazole
Hph	homophenylalanine
HRMS	high resolution mass spectrometry
^{1}H-NMR	proton nuclear magnetic resonance
I	inhibitor

iBCF	isobutyl chloroformate
Ile	isoleucine
IrAE	*Ixodes ricinus* asparaginyl endopeptidase
k	rate constant
k_{cat}	catalytic constant (s^{-1})
K_i	inhibition constant
k_{inact}	inactivation constant
k_{obs}	observed rate constant
KOH	potassium hydroxide
Leu	leucine
Lys	lysine
M	molarity (moles/liter)
Me-N-Piz	N-methylpiperazine-CO
MeOH	methanol
MHz	megahertz
$MgSO_4$	magnesium sulfate
min	minutes
mM	millimolar
ml	milliliter
MS	mass spectrometry
Mu	morpholine-CO
$NaBH_3CN$	sodium cyanoborohydride
NaCN	sodium cyanide
$NaHCO_3$	sodium bicarbonate
Naphth	naphthyl

Na_2SO_4	sodium sulfate
N.I.	no inhibition
NMM	*N*-methylmorpholine
OBzl	benzyloxy
Pd	palladium
Ph	phenyl
Phe	phenylalanine
Pip	piperidine-CO
Piz	piperazine-CO
ppm	parts per million
s	second
S	substrate
SAR	structure activity relationships
SmAE	*Schistosoma mansoni* asparaginyl endopeptidase
Suc	succinyl
t	time
TFA	trifluoroacetic acid
THF	tetrahydrofuran
Tris	tris(hydroxymethyl)aminomethane
Trp	tryptophan
v	velocity
Val	valine
VS	vinyl sulfone

SUMMARY

Cysteine proteases are important drug targets due to their involvement in many biological processes such as protein turnover, digestion, blood coagulation, apoptosis, cell differentiation, cell signaling, and the immune response. In this thesis, we have reported the design, synthesis and evaluation of clan CA and clan CD cysteine protease inhibitors.

In the first project, we focused on the design, synthesis and evaluation of aza-peptidyl Michael acceptor and epoxide inhibitors for asparaginyl endopeptidases (legumains) from the bloodfluke, *Schistosoma mansoni* (SmAE) and the hard tick, *Ixodes ricinus* (IrAE). Structure activity relationships (SARs) were determined for a set of forty one aza-peptide Michael acceptors and eight aza-peptide epoxides. For both enzymes, SARs were similar, but with some notable exceptions. Both enzymes prefer disubstituted amides to monosubstituted amides in the P1' position and potency increased as we increased the hydrophobicity of the inhibitor in this position. Extending the inhibitor to P5 resulted in increased inhibitory potency, especially against IrAE, and both enzymes prefer small over large hydrophobic residues in the P2 position. Aza-peptide Michael acceptor inhibitors are more potent than aza-peptide epoxide inhibitors and, for some of these compounds, second order inhibition rate constants are the fastest yet discovered.

For the second project, we synthesized seventeen aza-peptidyl Michael acceptor and epoxide inhibitors for the parasitic cysteine proteases; cruzain, rhodesain and TbCatB. We have found that monosubstituted amides were favored over disubstituted amides indicating the involvement of the amide hydrogen in a H-bond network. We have

shown that aza-peptide epoxides were as potent as Michael acceptors and we have obtained compounds with IC_{50} values as low as 20 nM.

In the third project, we worked on the synthesis of heterocyclic peptidyl α-ketoamides, peptidyl ketones and aza-peptidyl ketones as calpain inhibitors. We have synthesized peptidyl α-ketoamides with nucleotide bases in the primed region to create compounds that can cross the blood-brain barrier. Based on the crystal structure obtained with previous heterocyclic peptidyl α-ketoamides synthesized by our group, we have improved the potency by introducing a hydrophobic group on the adenine ring. We have obtained compounds with K_i values in the nanomolar range. We have also designed two new classes of inhibitors for calpain; peptidyl aminoketones and aza-peptidyl ketones. Peptidyl aminoketones were less potent than peptidyl α-ketoamides but still reasonable inhibitors of calpain that have the potential to cross the BBB. Aza-peptidyl ketones, on the other hand, did not inhibit calpain and cathepsin B.

CHAPTER 1

INTRODUCTION

PROTEASES

Proteases or proteolytic enzymes catalyze the breakdown of proteins by hydrolysis of peptide bonds. Proteases were first organized as clans and families by Rawlings and Barrett in 1993.[1] Proteases use different catalytic mechanisms for substrate hydrolysis. In the MEROPS database, proteases are divided into eight groups: serine, cysteine, threonine, aspartic, metallo, glutamic, mixed and unknown. Each group is further divided into families and clans according to their similarities in structure and evolutionary origin.

Proteases specifically cleave protein substrates either in the middle of the molecule (endopeptidases) and/or from the N or C termini (aminopeptidases and carboxypeptidases, respectively).

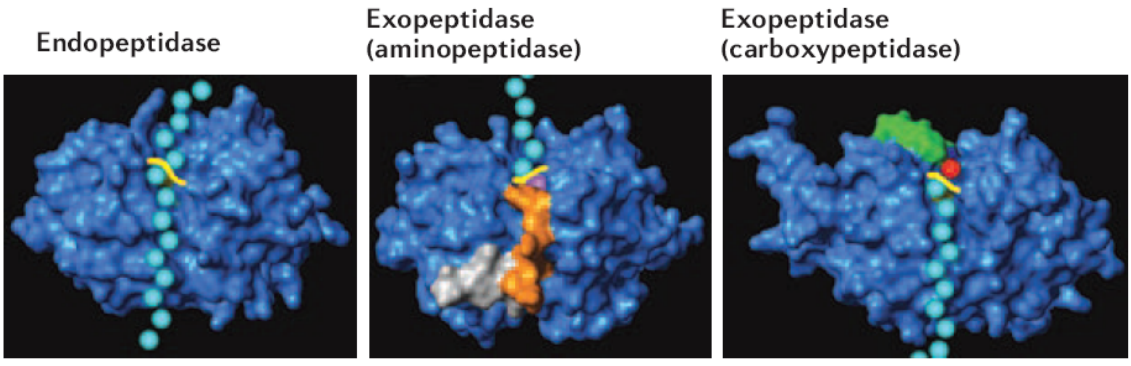


Figure 1.1. Modes of Substrate Cleavage by Peptidases[2]

Proteases are found in all organisms in the animal kingdom and involved in important physiological processes including cell differentiation and proliferation, cell death, DNA replication, tissue remodelling, wound healing, the immune response, protein turnover, digestion and signal transduction. Dysregulation of protease activities have been associated with many diseases including cardiovascular and inflammatory diseases, cancer, osteoporosis and neurological. Proteases are ideal targets for the development of selective inhibitors because of their involvement in a variety of disease states.

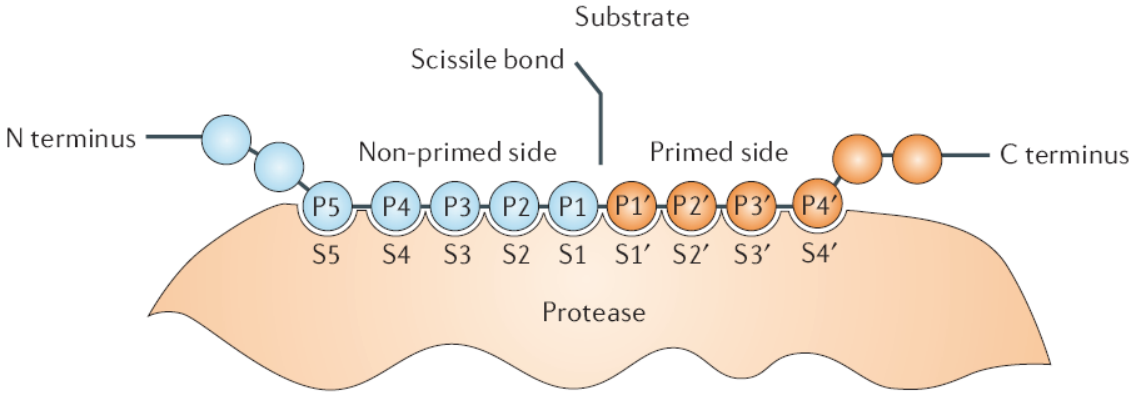


Figure 1.2. Nomenclature of Protease Subsites[2]

The nomenclature of protease subsites are first described by Berger and Schechter.[3] The surface of the protease that accommodates a single side chain of a substrate residue is called the subsite. Subsites are numbered S1, S2, etc. upwards towards the N terminus of the substrate (non-primed sites), and S1′, S2′, etc. towards the C terminus (primed sites), beginning from the sites on each side of the scissile bond. The substrate residues they accommodate are numbered P1, P2, etc, and P1′, P2′, etc.;

respectively. Substrate specificity of a protease, which is determined by the structure of the active site of the protease, determines which substrate residues can bind to specific substrate binding sites of the protease.

CYSTEINE PROTEASES

Cysteine proteases have a cysteine residue in their active side and therefore employ the sulfhydryl group of the side chain of a cysteine residue as the catalytic nucleophile. The first step of the peptide hydrolysis mechanism involves the deprotonation of the sulfhydryl group in the enzyme's active site by the histidine residue. A catalytic dyad is formed with the imidazole side chain of histidine residue and the sulfhydryl group of the active site cysteine. Some of the cysteine proteases use an extra amino acid to provide additional stabilization via hydrogen bonds, thus forming a catalytic triad. The next step is the nucleophilic attack of the thiolate anion to the carbonyl carbon of the peptide bond to be cleaved and a tetrahedral intermediate is formed. The tetrahedral intermediate is stabilized by the oxyanion hole via hydrogen bonds to the backbone amides. Collapse of the tetrahedral intermediate releases the amino terminus of the substrate and results in the formation of an acyl intermediate. Abstraction of a proton from a water molecule by the catalytic histidine forms an activated water molecule. The thioester bond of the acyl intermediate is then hydrolyzed by the activated water molecule and the free enzyme is regenerated. (Figure 1.3)

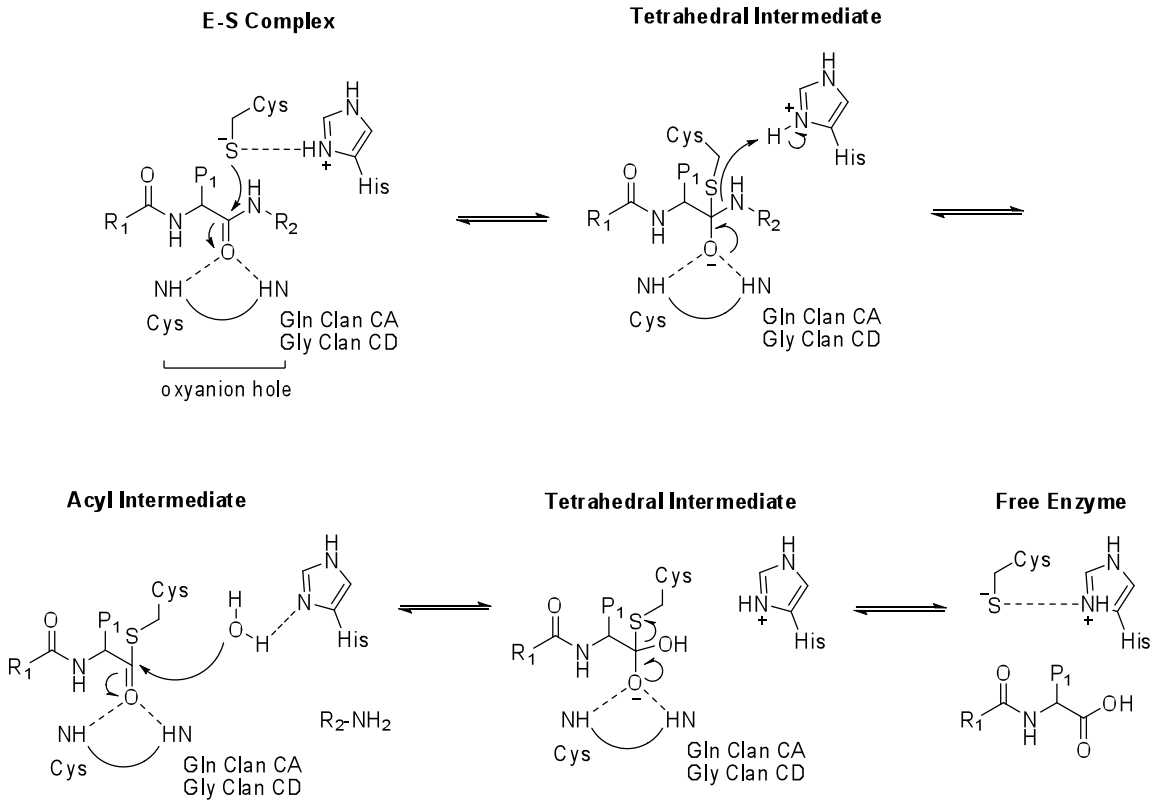


Figure 1.3. Substrate Hydrolysis Mechanism of Cysteine Proteases

Cysteine proteases currently consist of 10 clans. We will focus on clan CA and clan CD cysteine proteases since the inhibitors reported in this thesis are designed for the enzymes belong to these two clans. Clan CA and clan CD cysteine proteases differ slightly in their substrate hydrolysis mechanism. A catalytic dyad of Cys and His is employed by clan CD whereas a catalytic triad of Cys, His, and Asn is used by clan CA cysteine proteases. Stabilization of the tetrahedral intermediate occurs by H bonding to the backbone NH of active site Cys and to the NH_2 group of the Gln side chain in clan CA.[4] The oxyanion hole consists of only backbone NH groups in clan CD cysteine proteases.

Clan CA Cysteine Proteases

The majority of cysteine proteases belong to clan CA and these cysteine proteases have been found in viruses, bacteria, protozoa, plants and mammals.[5] Clan CA contains all the families of peptidases that have similar structures to papain.

Papain is a cysteine protease extracted from the tropical papaya fruit. It is the first cysteine protease to be discovered and has been the subject of mechanism and structural studies for many years and it is also the first enzyme to have its crystal structure determined.[6] The substrate specificity of clan CA enzymes is primarily controlled by the S2 subsite. Papain has a very broad specificity compared to other clan CA proteases. It prefers bulky hydrophobic residues in the P2 position, while the S1 subsite is not as selective as the S2 subsite, but has some preference for Arg and Lys over other residues such as Val.[7]

Cathepsin B is a lysosomal cysteine protease with both endopeptidase and dipeptidyl carboxypeptidase activities. The substrate specificity of cathepsin B is similar to papain family and the primary determinant of specificity is the S2 subsite. Cathepsin B can accommodate hydrophobic residues together with arginine in the P2 position due to the location of Glu245 in the S2 subsite.[8] Cathepsin B is implicated in diseases such as arthritis, muscular dystrophy, gingivitis and cancer.[9]

Calpains are a unique family of cysteine proteases since they require calcium to become activated. The major isoforms of calpain (calpain I and calpain II) are nearly identical and differ mostly in the amount calcium they require for activation.[10] Calpain activation is utilized in many cellular processes such as apoptosis, cell differentiation and protein turnover.[11] Calpains prefer hydrophobic residues like Val or Leu in the P2

position but are less selective in the P1 position.[12] Overactivation of calpain has been observed in a variety of disorders, including cataracts, muscular dystrophy, cancer and neurodegenerative diseases.[13]

Cruzain is the only parasitic cysteine protease with a crystal structure.[14-16] Cruzain is expressed in all life cycles of *Trypanasoma cruzi*,[5] which is the causative agent of Chagas' disease in Central America. Cruzain can accommodate hydrophobic residues like phenylalanine together with basic residues such as arginine in the S2 pocket. Cruzain is an excellent target for the development of irreversible inhibitors due to the presence of the enzyme in all stages in the life cycle of the parasite.

Rhodesain is the major cysteine protease of *T. brucei rhodesiense*, the causative agent of African sleeping sickness.[17] Rhodesain is an important target for the development of antiparasitic chemotherapy due to the involvement of the enzyme in regulating the replication of the parasite. Therefore, inhibition of the enzyme will block the life cycle of the parasite in infected mammalian cells.

TbCatB has recently been identified in *T. brucei* as a key enzyme in host protein turnover and iron acquisition.[18] TbcatB, a cathepsin B-like enzyme, is an ideal target for the development of new anti-trypanosomal chemotheraphy.

Clan CD Cysteine Proteases

Clan CD is a smaller class of cysteine proteases compared to clan CA cysteine proteases. Clostripain (C11), legumains (C13), caspases (C14), gingipain (C25), separase (C50) and self-cleaving toxin A (C80) belong to clan CD cysteine proteases. The substrate specificity of the members of this class is determined by the residue in the P1

position, such as Asp for caspases, Arg for clostripain, Asn for legumain family, Arg or Lys for gingipain family, Arg for separase family and Leu for self-cleaving toxin A family. Unlike clan CA cysteine proteases, clan CD enzymes employ a catalytic dyad (His-Cys) for substrate hydrolysis.

Asparagine endopeptidases (legumains) cleave their peptide substrates specifically after an asparagine residue. Asparagine endopeptidases (AEs) have been linked to osteoclast formation and bone resorption,[19] processing of bacterial antigens[20] and elevated levels of human AE was found in many tumors, including carcinomas of the breast, colon, and prostate.[21] AE of the bloodfluke *Schistosoma mansoni* and AE of the hard tick *Ixodes ricinus* have an indirect role in host hemoglobin digestion[22, 23] and therefore represent important targets for the development of inhibitors.

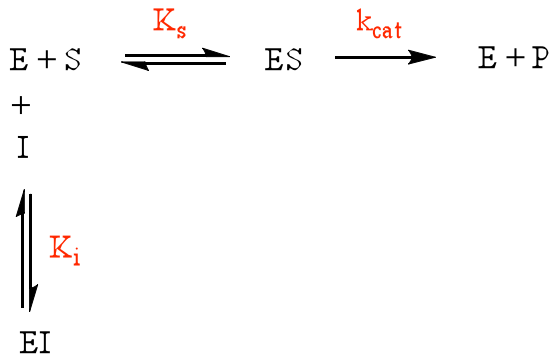
ENZYME INHIBITION

An inhibitor is a compound that decreases the rate of an enzyme catalyzed reaction and this inhibition can be either irreversible or reversible depending on the nature of binding of inhibitor to the enzyme.

Reversible enzyme kinetics

Reversible inhibition can be competitive, uncompetitive or linear mixed type. The inhibitors studied in this thesis are competitive inhibitors of cysteine proteases since they are competing with the substrate for binding to the active site cysteine residue. The equations for the competitive inhibition are derived by using the following model. In this

model, E is the enzyme, S is the substrate, I is the inhibitor, P is the product of the hydrolysis reaction, ES is the enzyme-substrate complex and EI is the enzyme-inhibitor complex.



K_s is the enzyme-substrate dissociation constant, k_{cat} is the rate of formation of product from ES complex and K_i is the inhibition constant or enzyme-inhibitor dissociation constant.

$$K_s = ([E][S])/[ES]$$

$$K_i = ([E][I])/[EI]$$

$$v = k_{cat}\,[ES]$$

K_i can be determined from the Michaelis-Menten equation and equal to

$$K_i = (V_{max}\,[S])/(K_m\,(1 + [I]/K_i) + [S])$$

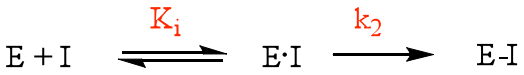
This equation can be rearranged as

$$1/v = [(1/V_{max})\,(1 + K_m/[S])] + [([I]\,K_m)/(V_{max}\,K_i\,[S])]$$

The plot of 1/v against [I] at constant [S], which is called Dixon plot,[24] will be a straight line. K_i can be easily calculated based on this equation; when rates are measured at many different inhibitor concentrations but only a limited number of substrate concentrations. Low K_i values indicate the higher potency of the inhibitor.

Irreversible enzyme kinetics

Irreversible inhibition of an enzyme proceeds through the formation of an intermediate non-covalent enzyme-inhibitor complex (E•I) and which is then followed by the formation of a covalent enzyme-inhibitor complex (E-I) in a time-dependent manner.



E is the enzyme, I is the inhibitor, K_i is the dissociation constant of the non-covalent enzyme-inhibitor complex and k_2 is the first order rate constant for the formation of covalent enzyme-inhibitor complex.

One of the methods used for the determination of irreversible enzyme kinetics, developed by Kitz and Wilson, is called the incubation assay method.[25] In this method, enzyme and a large excess of inhibitor are incubated together in buffer, and then an aliquot of this solution is diluted into a buffer solution containing the substrate and the rate of substrate hydrolysis is measured. When [I] > [E], pseudo-first order kinetics can be assumed and the rate can be calculated from the equation,

$$\ln (v_t/v_0) = -k_{obs}t$$

where v_0 is the initial rate of hydrolysis without the presence of the inhibitor and v_t is the rate of hydrolysis at time t, k_{obs} is the pseudo-first-order rate constant for inactivation. Pseudo-first-order kinetics are also described by the equation,

$$k_{obs} = k_2/(1 + K_i/[I])$$

This equation can be rearranged as

$$1/k_{obs} = K_i/k_2[I] + 1/k_2$$

where K_i ($K_i = [E][I]/[E \cdot I]$) is the dissociation constant of the enzyme-inhibitor complex. The rate constant k_2 is a first-order rate constant, and k_2/K_i is a second order rate constant and the most commonly used parameter to report inhibition data.

For very potent inhibitors where rates are too fast to measure using the pseudo-first order kinetics, the inhibitor concentration is decreased until $[I] \approx [E]$, then second-order rate constant can be calculated from the equation,

$$k_{2nd}t = [1/(i - e)]\ln[e(i - x)/i(e - x)]$$

where $e - x$ is the residual enzyme concentration and i is the initial inhibitor concentration. Since inhibitor concentration is included in the calculation, the second-order rate constant k_{2nd} can be determined from the equation.

Another method, developed by Tian and Tsou,[26] is used for the measurement of irreversible inhibition rates of very fast inhibitors. In this method, substrate hydrolysis is measured continuously upon addition of enzyme to a mixture of substrate and inhibitor. This method is referred to as the progress curve method and the reaction in the progress curve method is initiated by the addition of the enzyme. The observed inhibition rate constant k_{obs} can be calculated from the equation where [P] and $[P_\infty]$ are the product concentrations at t and $t = \infty$; respectively.

$$\ln ([P_\infty] - [P]) = \ln [P_\infty] - k_{obs}[I]t$$

The observed inhibition rate constant k_{obs} can be easily calculated from the slope of the plot $\ln ([P_\infty] - [P])$ versus t at a particular substrate concentration. The progress curve method has the advantage of consuming less enzyme and substrate since relatively few separate kinetic measurements are needed.

The inhibitor concentration required to decrease the activity of the enzyme by 50% is called the IC_{50} value of the inhibitor. The incubation time is an important factor for IC_{50} assays of irreversible inhibitors since a longer incubation time resulted in a lower IC_{50} value. It is possible to establish a mathematical relationship between the pseudofirst order inactivation rate k_{obs} and IC_{50} values. If it is assumed that IC_{50} is the inhibitor concentration necessary to reduce the enzyme activity by 50% during the time of the incubation, then the assay time (t_{assay}) should be equal to the half-life $t_{1/2}$ for the first order inactivation rate.

$$k_{obs} = \ln 2/t_{1/2} = 0.693/t_{assay}$$

$$k_{obs}/[I] = 0.693/(t_{assay} \times IC_{50})$$

CHAPTER 2

AZA-PEPTIDYL MICHAEL ACCEPTORS AND EPOXIDES AS INHIBITORS OF *SCHISTOSOMA MANSONI* AND *IXODES RICINUS* LEGUMAINS (ASPARAGINYL ENDOPEPTIDASES)

INTRODUCTION

The helmintic disease, schistosomiasis is the second most important parasitic disease after malaria in tropical areas. It infects over 200 million people in approximately 70 countries, with 779 million at risk, 85% of who are in Africa alone.[27]

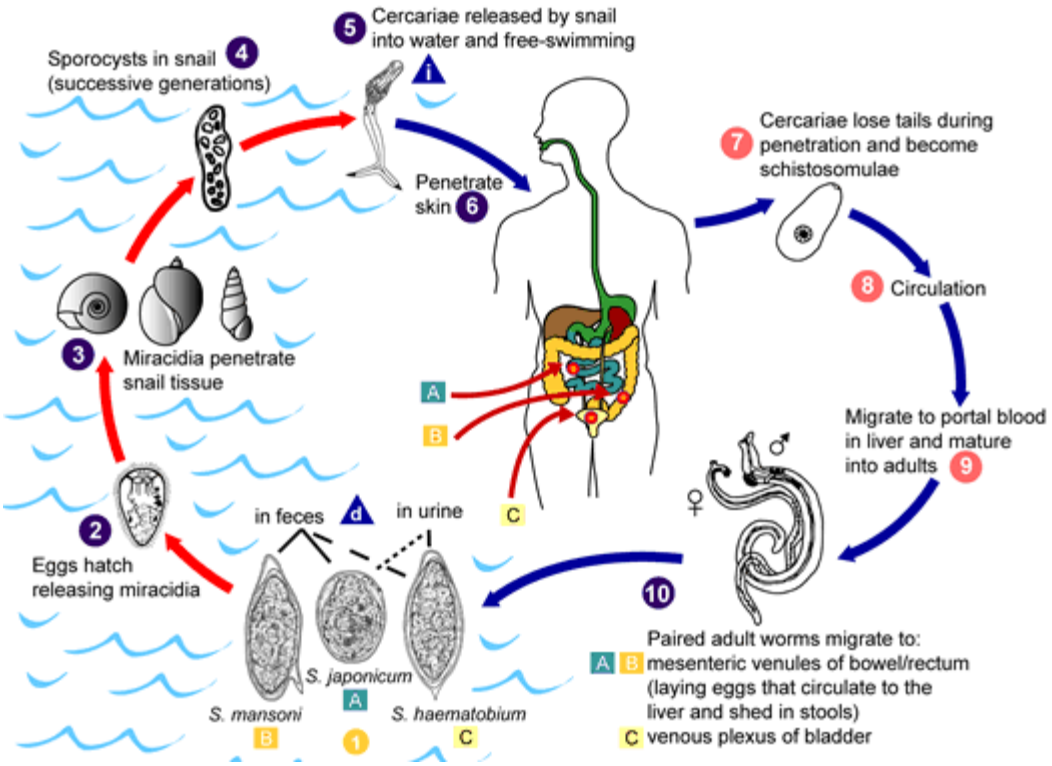


Figure 2.1. Life Cycle of Schistosome[28]

Immature and adult bloodflukes live in the cardiovascular system, ingest red blood cells and utilize hemoglobin as a source of amino acids for growth, development and reproduction. In the parasite gut, cysteine and aspartic proteases contribute to the proteolytic degradation of hemoglobin.[29] Among these are a legumain (SmAE) that, *in vitro*, degrades hemoglobin[30] and *trans*-activates the major gut cathepsin B protease.[31] As a central digestive enzyme, therefore, SmAE may represent a useful target for selective inhibitors that impair the parasite's ability to feed.

Hard ticks of the genus *Ixodes* are vectors of Lyme disease caused by the spirochetes *Borrelia burgdorferi sensu lato.* The multiplication of spirochetes in the tick gut and subsequent infection of the host requires unimpaired bloodmeal uptake and digestion.[32] As for SmAE, a gut-associated AE in *Ixodes ricinus* (IrAE) is thought to contribute to hemoglobin degradation either directly or indirectly by the *trans*-activation of other digestive cysteine and aspartic proteases.[33, 34] Accordingly, IrAE may represent a drug or vaccine target to prevent or retard transmission of disease.

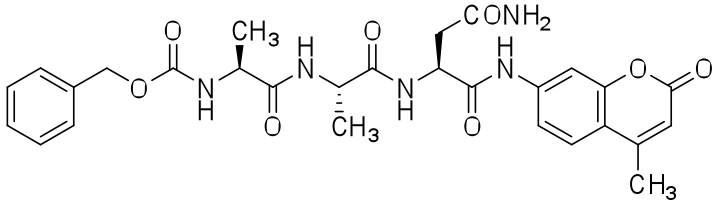
Asparaginyl endopeptidases (AEs) or legumains (EC.3.4.22.34) belong to the C13 family of clan CD cysteine proteases which also includes caspases, gingipains, clostripain, and separase.[35] AEs are acidic lysosomal enzymes that cleave substrates specifically after an asparagine residue in the P1 position.[36] Legumains were first discovered in the human blood fluke, *Schistosoma mansoni*,[37] then in plants,[38] mammals,[39-41] and most recently in the ticks, *Ixodes ricinus*[33] and *Haemaphysalis longicornis*.[42] Plant legumain functions as a processing enzyme of storage proteins during seed germination.[43] Mammalian legumain is involved in the inhibition of osteoclast formation and bone resorption.[44] It has also been shown that human legumain

is highly expressed in many tumors, including carcinomas of the breast, colon, and prostate.[21] Recently, it was found that mice lacking legumain develop disorders resembling hemophagocytic syndrome.[45]

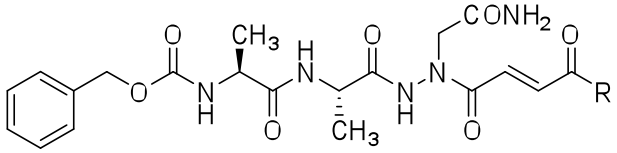
Synthetic AE inhibitors that have been tested against mammalian AEs[46, 47] include aza-Asn halomethylketones (Cbz-Ala-Ala-AAsn-CH_2Cl, $k_{obs}/[I]$ = 139,000 $M^{-1}s^{-1}$), Michael acceptors derived from Asn (Cbz-Ala-Ala-$NHCH(CH_2CONH_2)CH{=}CH_2CO_2CH_2CH{=}CH_2$, $k_{obs}/[I]$ up to 766 $M^{-1}s^{-1}$), and acyloxymethylketones ($k_{obs}/[I]$ from 769 up to 109,000 $M^{-1}s^{-1}$). It has also been shown that the aspartyl peptidyl fluoromethyl ketones designed for caspase inhibition also moderately inhibit mammalian AE.[48] It has been recently reported that aza-asparaginyl Michael acceptor inhibitors are potent and selective inhibitors of *S. mansoni*, *I. ricinus* and *Trichomonas vaginalis* (a parasitic protozoan) AEs.[49] In this study, the inhibitor sequence Cbz-Ala-Ala-AAsn was maintained while substituents on the reactive warhead were changed in order to measure the effects of modifying the P1' position. SAR studies revealed that the most potent inhibitors have esters or disubstituted amides with aromatic groups in the P1' position.

INHIBITOR DESIGN

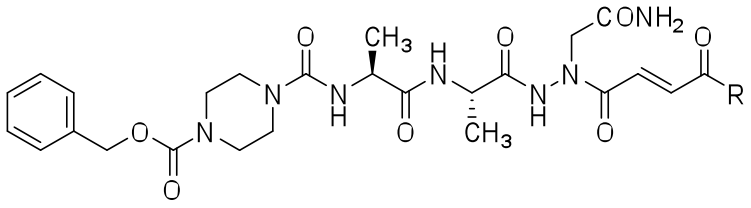
Aza-peptide Michael acceptors and epoxide inhibitors are selective and potent inhibitors of SmAE and IrAE.[49, 50] Previously synthesized inhibitors have the peptide sequence of Cbz-Ala-Ala-AAsn as a template since Cbz-Ala-Ala-Asn-NHMec (NHMec = 7-(4-methyl)coumarylamide) is an optimal substrate sequence for legumain.[51]



Cbz-Ala-Ala-Asn-AMC



Cbz-Ala-Ala-AAsn-CH=CH-COR



Cbz-Piz-Ala-Ala-AAsn-CH=CH-COR

Figure 2.2. Design of Aza-peptide Epoxide and Michael Acceptors.

In this study, we used Boc-NHNHCH$_2$CONH$_2$, YCO-Ala-NHNHCH$_2$CONH$_2$ and YCO-Ala-Ala-NHNHCH$_2$CONH$_2$ peptide sequences where the Y groups are piperidine, morpholine, piperazine, *tert*-butoxypiperazine and benzyloxycarbonyl piperazine. These groups were chosen to increase the bioavailability of the inhibitors and to study the interactions of the P4 (YCO-Ala-AAsn-CH=CH-COR) and P5 (YCO-Ala-Ala-AAsn-CH=CH-COR, YCO-Ala-Ala-AAsn-EP-COR) positions of the inhibitor with SmAE and IrAE. Work by other investigators with the vinyl sulfone inhibitors of cysteine proteases demonstrated that introduction of the appropriate N-terminal capping groups could

increase compound bioavalibitiy.[52] Accordingly, we expanded upon our previous design of aza-peptide Michael acceptors and epoxides by introducing groups at the N-terminus in order to both extend the compounds to P4 and P5 and potentially increase bioavailability.

For the inhibitor design, the α-carbon of the asparagine (Asn) residue is replaced by a nitrogen atom. This replacement results in an aza-peptide containing the aza-asparagine (AAsn) residue. Presence of an aza-asparagine residue at P1 makes the synthesis of aza-peptide Michael acceptor and epoxide inhibitors easier compared to peptide Michael acceptor and epoxide inhibitors as the synthesis involves coupling of an acid and an aza-peptide precursor. Aza-peptides are ideal inhibitors because they are resistant to cleavage by proteases *in vivo*[53, 54] and can incorporate a reactive warhead. We chose the epoxide and Michael acceptor double bonds as these warheads can be easily modified in the P' position to study interactions with the S' subsites of the enzymes.

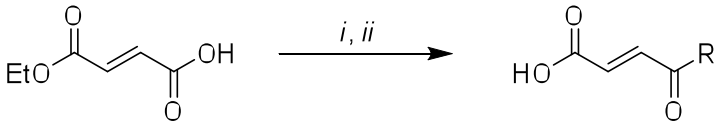
We have designed aza-peptide epoxides and Michael acceptor inhibitors that closely resemble an extended peptide legumain substrate.[55, 50] Placement of the carbonyl group of the epoxide moiety or fumarate derivative in the inhibitor places it in a location identical to that of the carbonyl of the scissile peptide bond in a legumain substrate. This design allows the peptide chain of the inhibitor to exactly match that of a good substrate from the N-terminus across to the scissile peptide carbonyl group. Aza-peptide epoxides and Michael acceptors have the advantage of being easily extended in the P' direction, allowing interactions with the S' subsites of AE.

We have also synthesized inhibitors with the Cbz-Ala-AA-AAsn peptide sequence where AA is valine, isoleucine or phenylalanine to study the effect of

modification in the P2 position. Aza-peptide substrate analogs were also synthesized where aza-peptide precursors were reacted with different isocyanates or coupled to an carboxylic acid.

CHEMISTRY

The synthesis of the aza-peptide fumarate and epoxide analogs are based on the previous syntheses of aza-peptide Michael acceptor[55] and epoxide inhibitors.[56] The inhibitors are obtained by coupling a peptidyl hydrazide to a monoester or amide of fumaric acidor to a monoester or amide of epoxysuccinic acid.



1 **2 a-p**

R=

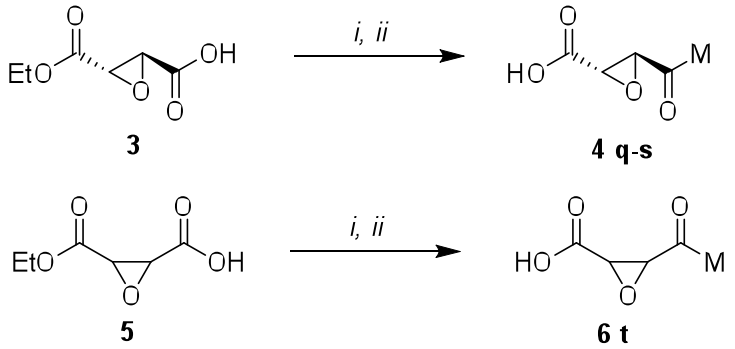
a = -OEt
b = -OBzl
c = -NH-cyclopropyl
d = -NHCH$_2$-cyclohexyl
e = -NHCH$_2$-2-furyl
f = -NHBzl
g = -NHCH$_2$CH$_2$Ph
h = -NHCH$_2$CH$_2$CH$_2$Ph
i = -NHCH$_2$Ph-3,4-OCH$_3$
j = -tetrahydroquinoline
k = -tetrahydroisoquinoline
l = -N(Bzl)$_2$
m = -NHCH$_2$-1-naphthyl
n = -N(CH$_3$)CH$_2$-1-naphthyl
o = -N(Bzl)-CH$_2$-1-naphthyl
p = -N(CH$_2$-1-naphthyl)$_2$

Reagents: (*i*) NMM, iBCF, CH_2Cl_2, HNR_1R_2; (*ii*) 1. NaOH, 2. HCl, EtOH.

Figure 2.3. Synthesis of Fumarate Precursors.

Monoethyl fumarate (**2a**) was commercially available and all the Michael acceptor warheads were synthesized using this compound as a precursor. The monobenzyl fumarate (**2b**) was formed from monoethyl fumarate and benzyl alcohol using NMM and DCC as the coupling reagent and followed by deprotection of the ethyl

ester in ethanol using aqueous NaOH. The fumarate precursors (**2c - p**) were prepared from monoethyl fumarate and the corresponding primary or secondary amines by standard mixed anhydride coupling using NMM and iBCF; and followed by hydrolysis of the ethyl ester with aqueous NaOH (Figure 2.3).



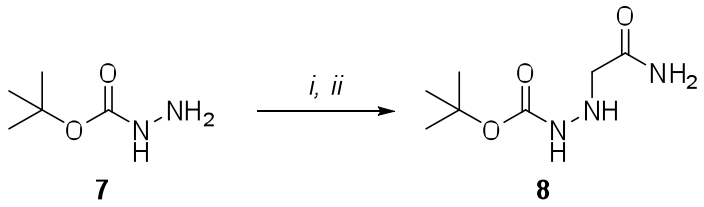
Reagents: (*i*) NMM, iBCF, CH_2Cl_2, HNR_1R_2; (*ii*) 1. KOH, 2. HCl, EtOH.

Figure 2.4. Synthesis of Epoxysuccinate Derivatives.

A previously described procedure[56] was used for the synthesis of (*2S,3S*)-oxirane-2,3-dicarboxylic acid monoethyl ester (**3**). The epoxide warheads (**4r, 4s**) were synthesized from monoethyl epoxysuccinate and the corresponding primary or secondary amines using the standard mixed anhydride coupling procedure with NMM and iBCF, followed by deprotection of the ethyl ester in ethanol using aqueous KOH (Figure 2.4). The monoethyl ester of *cis*-oxirane-2,3-dicarboxylic acid monoethyl ester (**5**) was synthesized following a previously described procedure.[57] Coupling of the

dibenzylamine to the monoethyl ester epoxysuccinate to form compound 6t was performed using the standard mixed anhydride coupling procedure and followed by deprotection as decribed above. The disubstituted aromatic amines were synthesized by reductive amination starting with an aromatic aldehyde precursor and an aromatic primary amine.

The hydrazide Boc-NHNHCH$_2$CONH$_2$ was synthesized by the monoalkylation of *tert*-butyl carbazate (**7**) with ethyl bromoacetate and the conversion of the ethyl ester to the amide by ammonolysis was done with catalytic amounts of NaCN according to the procedure by Hogberg *et al* (Figure 2.5).[58]

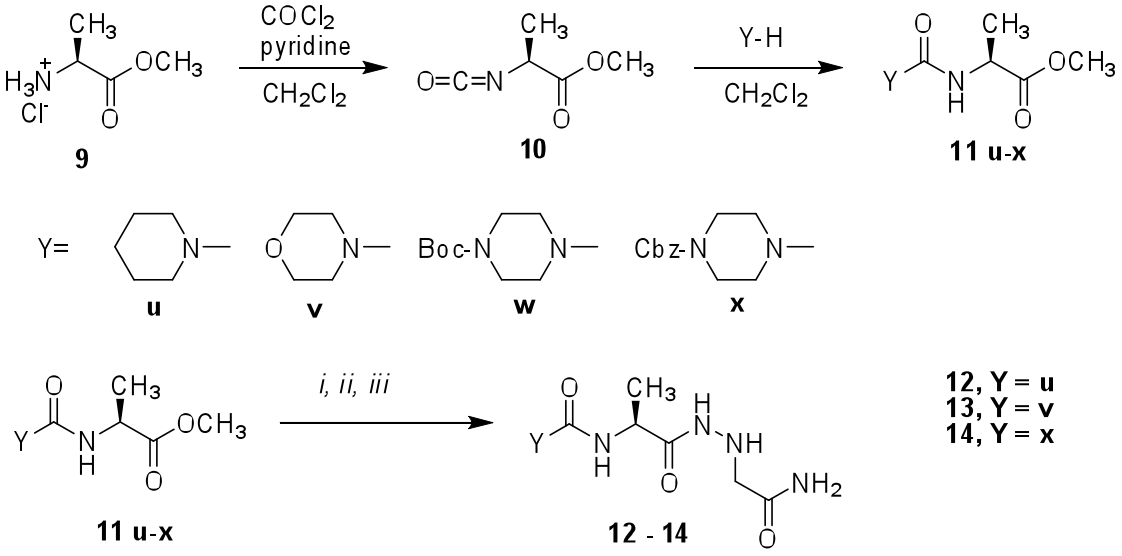


Reagents: (*i*) BrCH$_2$COOEt, NMM, DMF; (*ii*) NH$_3$, cat. NaCN, MeOH, DMF.

Figure 2.5. Synthesis of the Aza-asparagine Precursor.

For the synthesis of acyl dipeptide aza-asparagine precursors (**12 - 14**), we first prepared the alanine methyl ester isocyanate (**10**) by reacting the hydrochloride salt of alanine (**9**) with phosgene according to the procedure by Nowick *et al*.[59] Alanine methyl ester isocyanate (**10**) was then reacted with piperidine, morpholine, *tert*-butoxycarbonylpiperazine, benzyloxycarbonylpiperazine to form Y-CO-Ala-OCH$_3$ (**11u - x**) respectively.[60] Piperidine and morpholine were commercially available, however; *tert*-butoxycarbonylpiperazine[61] and benzyloxycarbonylpiperazine[62] were synthesized according to the literature. The ester Y-CO-Ala-OCH$_3$ (**11u - x**) was then reacted with

excess hydrazine (NH_2NH_2) in methanol to form Y-CO-Ala-NH-NH_2. To introduce the aza-asparagine side chain, Y-CO-Ala-NH-NH_2 was reacted with ethyl bromoacetate and NMM. The ethyl ester group was then reacted with 7N NH_3 in methanol in the presence of catalytic NaCN to form the acyl dipeptide aza-asparagine precursor (Y-CO-Ala-$NHNHCH_2CONH_2$) (Figure 2.6).

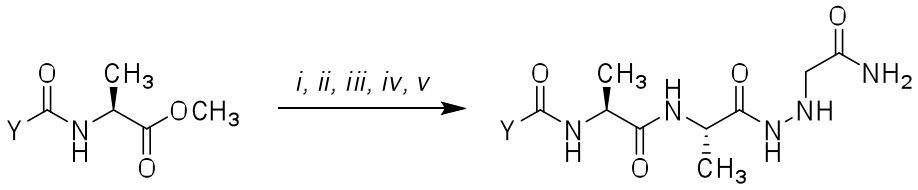


Reagents: (*i*) H_2NNH_2, MeOH; (*ii*) $BrCH_2COOEt$, NMM, DMF; (*iii*) NH_3, cat. NaCN, MeOH, DMF.

Figure 2.6. Synthesis of the Acyl Dipeptide Aza-asparagine Precursors.

For the synthesis of the acyl tripeptide aza-asparagine precursors (**15 - 18**), we first hydrolyzed the esters **11u – x** (Y-CO-Ala-OCH_3) with 1M NaOH in methanol to form acyl alanine derivatives (Y-CO-Ala-OH). The acyl alanine then was coupled to alanine methyl ester using HOBt and DCC to form the dipeptides Y-CO-Ala-Ala-OCH_3. The dipeptide esters Y-CO-Ala-Ala-OCH_3 were then reacted with excess hydrazine and

the asparagine side chain was introduced as mentioned previously to form the acyl tripeptide aza-asparagine precursors (Y-CO-Ala-Ala-NHNHCH$_2$CONH$_2$) (Figure 2.7).

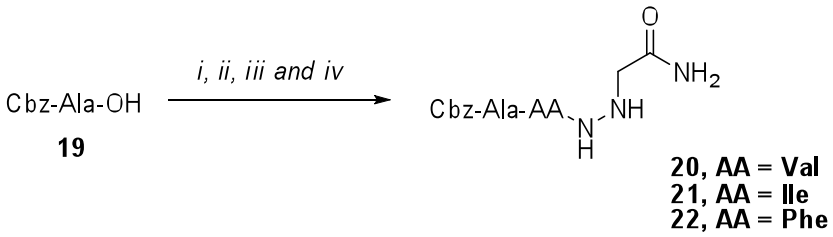


11 u-x **15 - 18**

Reagents: (*i*) 1. NaOH, 2. HCl; (*ii*) HCl·H$_2$N-Ala-OCH$_3$, HOBt, DCC, DMF; (*iii*) H$_2$NNH$_2$, MeOH; (*iv*) BrCH$_2$COOEt, NMM, DMF; (*v*) NH$_3$, cat. NaCN, MeOH, DMF.

Figure 2.7. Synthesis of the Acyl Tripeptide Aza-asparagine Precursors.

For the synthesis of Cbz-Ala-Val(Ile, Phe)-NHNHCH$_2$CONH$_2$, we reacted Cbz-Ala-OH with valine methyl ester, isoleucine methyl ester, or phenylalanine methyl ester. The peptidyl methyl ester was then reacted with hydrazine and finally the asparagine side chain was attached by alkylation with ethyl bromoacetate followed by ammonolysis (Figure 2.8).



19

Reagents: (*i*) HCl·H-Val-OCH$_3$, HCl·H-Ile-OCH$_3$ or HCl·H-Phe-OCH$_3$, HOBt, DCC, DMF (*ii*) H$_2$NNH$_2$, MeOH, (*iii*) BrCH$_2$COOEt, NMM, DMF; (i*v*) NH$_3$, cat. NaCN, MeOH, DMF.

Figure 2.8. Synthesis of Cbz-Ala-AA-NHNHCH$_2$CONH$_2$.

The aza-asparagine precursors (**8**, **12 – 14**, **15 – 18**, **20 - 22**) were then coupled to a variety of substituted fumarate analogs (**2a - p**), epoxide moieties (**4q – s** and **6t**) using HOBt and EDC to complete the synthesis of the aza-peptidyl inhibitors (Figure 2.9).

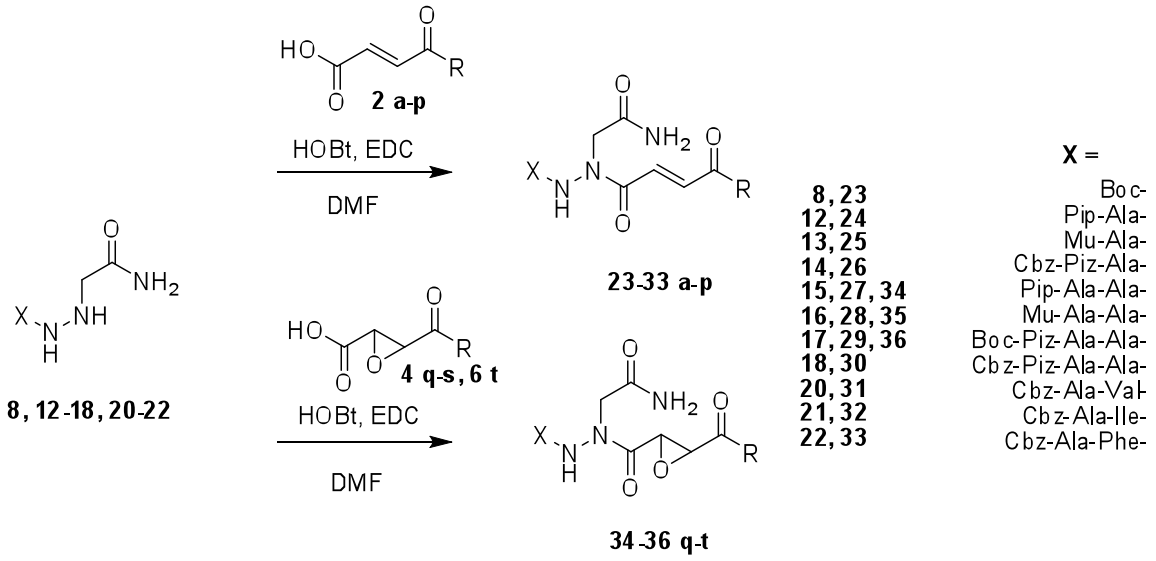


Figure 2.9. Coupling of Fumarate Precursors and Epoxide Moieties to Aza-asparagine Precursors.

Aza-peptide Michael acceptors and epoxides with Boc-Piz-Ala-Ala-AAsn peptide sequence (**29, 36 a - r**) were treated with TFA in dichloromethane to form the simple piperazine derivatives Piz-Ala-Ala-AAsn (**37, 38 a - r**).

Aza-peptidyl substrate analogs were synthesized in three different ways (Figure 2.10). Aza-peptide precursor (**13**) was reacted with hydrocinnamic acid using HOBt and EDC coupling procedure to form compound **39**. Different isocyanates were added to a solution of aza-peptide precursors in dichloromethane to form compounds (**40-42**). Aza-

peptide precursor (**16**) was reacted with benzylchloroformate in the presence of pyridine to form compound (**43**).

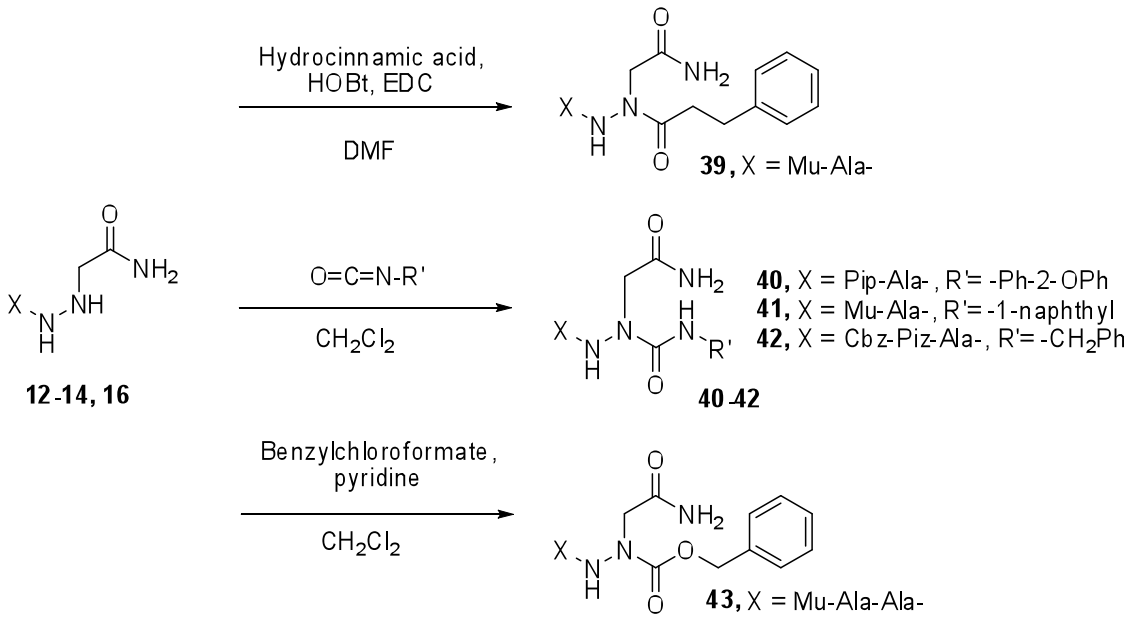


Figure 2.10. Synthesis of Aza-peptidyl Substrate Analogs.

RESULTS AND DISCUSSION

Inhibition of SmAE and IrAE with Aza-peptidyl Michael Acceptors. We synthesized four simple Boc-protected aza-asparagine inhibitors (**23a, d, f, h**) with different Michael acceptor warheads. Only the monoethyl ester derivative (SmAE IC_{50} > 2,000 nM, IrAE IC_{50} = 750 nM) inhibited the enzymes, whereas no inhibition was detected with the other three compounds (Table 2.1). Clearly, inhibitors with a single aza-amino acid residue are not very effective.

Table 2.1. Inhibition of SmAE and IrAE by Michael Acceptors with the Boc-AAsn Sequence.

	Inhibitor	SmAE IC_{50} (nM)	IrAE IC_{50} (nM)
23a	Boc-AAsn-CH=CH-COOEt	>2,000	750
23d	Boc-AAsn-CH=CH-$NHCH_2$-cyclohexyl	N.I.	N.I.
23f	Boc-AAsn-CH=CH-CONHBzl	N.I.	N.I.
23h	Boc-AAsn-CH=CH-$CONHCH_2CH_2CH_2Ph$	N.I.	N.I.

Due to this result, we next synthesized acyl dipeptide aza-asparagine Michael acceptor inhibitors. The results are shown in Table 2.2. Monosubstituted amides (**24k, 25c, e, i, h, k, 26j, k**) were either poor or non-inhibitory. Replacement of the monosubstituted amides with the disubstitued amides (**24k**, **25k, 26j - k**) yielded increased potency. Inhibitors **24k**, **25k**, and **26k** have the same isoquinoline moiety in their P1' position, however, they have different acyl groups. Both enzymes seem to favor the presence of a piperidine residue in the P3 position rather than morpholine or benzyloxypiperazine. Probably, the oxygen of the morpholine is disturbing the H-bond network around the active site of the AEs, whereas the benzyloxypiperazine is not accommodated in the P3 position due to the bulkiness of the group. Although the disubstituted amides were better inhibitors than the monosubstituted ones, they were still weak.

Table 2.2. Inhibition of SmAE and IrAE by Acyl Dipeptide Michael Acceptors.

	Inhibitor	SmAE IC_{50} (nM)	IrAE IC_{50} (nM)
24k	Pip-Ala-AAsn-CH=CH-CO-tetrahydroisoquinoline	600	150
25c	Mu-Ala-AAsn-CH=CH-CONH-cyclopropyl	N.I.	N.I.
25e	Mu-Ala-AAsn-CH=CH-CONHCH_2-2-furyl	N.I.	N.I.
25i	Mu-Ala-AAsn-CH=CH-CONHCH_2Ph-3,4-OCH_3	>2,000	>2,000
25h	Mu-Ala-AAsn-CH=CH-CONH$CH_2CH_2CH_2$Ph	>2,000	N.I.
25k	Mu-Ala-AAsn-CH=CH-CO-tetrahydroisoquinoline	750	600
26j	Cbz-Piz-Ala-AAsn-CH=CH-CO-tetrahydroquinoline	750	850
26k	Cbz-Piz-Ala-AAsn-CH=CH-CO-tetrahydroisoquinoline	750	800

In view of these results, acyl tripeptide aza-asparagine Michael acceptors were synthesized. The results are shown in Table 2.3. Among the inhibitors with Pip-Ala-Ala-AAsn sequence, the ethyl ester derivative (**27a**) was as effective (SmAE IC_{50} = 80 nM, IrAE IC_{50} = 15 nM) as the dibenzyl amide analog (**27l**) (SmAE IC_{50} = 80 nM, IrAE IC_{50} = 17 nM). Again, the disubstituted amides (**27l**, **o**, **p**) were more potent than the monosubstituted amide analog (**27g**). The dinaphthylmethylene derivative (**27p**) was the most potent of the acyl tripeptide inhibitors against SmAE (IC_{50} = 57 nM), whereas the N-benzyl-N-naphthylmethylene derivative was best against IrAE (IC_{50} = 2.4 nM).

Table 2.3. Inhibition of SmAE and IrAE by Acyl Tripeptide Michael Acceptors.

	Inhibitor	SmAE IC_{50} (nM)	IrAE IC_{50} (nM)
27a	Pip-Ala-Ala-AAsn-CH=CH-COOEt	80	15
27g	Pip-Ala-Ala-AAsn-CH=CH-CONHCH$_2$CH$_2$Ph	750	400
27l	Pip-Ala-Ala-AAsn-CH=CH-CON(Bzl)$_2$	80	17
27o	Pip-Ala-Ala-AAsn-CH=CH-CON(Bzl)-CH$_2$-1-naphthyl	100	2.4
27p	Pip-Ala-Ala-AAsn-CH=CH-CON(CH$_2$-1-naphthyl)$_2$	57	8
28a	Mu-Ala-Ala-AAsn-CH=CH-COOEt	101	0.23
28h	Mu-Ala-Ala-AAsn-CH=CH-CONHCH$_2$CH$_2$CH$_2$Ph	1450	>2,000
28k	Mu-Ala-Ala-AAsn-CH=CH-CO-tetrahydroisoquinoline	103	65
28p	Mu-Ala-Ala-AAsn-CH=CH-CON(CH$_2$-1-naphthyl)$_2$	45	3.3
37a	Piz-Ala-Ala-AAsn-CH=CH-COOEt	300	8.5
37b	Piz-Ala-Ala-AAsn-CH=CH-COOBzl	250	13
37l	Piz-Ala-Ala-AAsn-CH=CH-CON(Bzl)$_2$	90	31
37n	Piz-Ala-Ala-AAsn-CH=CH-CON(CH$_3$)CH$_2$-1-naphthyl	40	3.8
37p	Piz-Ala-Ala-AAsn-CH=CH-CON(CH$_2$-1-naphthyl)$_2$	70	2.6
29a	Boc-Piz-Ala-Ala-AAsn-CH=CH-COOEt	80	8.5
29b	Boc-Piz-Ala-Ala-AAsn-CH=CH-COOBzl	45	7.5
29l	Boc-Piz-Ala-Ala-AAsn-CH=CH-CON(Bzl)$_2$	45	30
29n	Boc-Piz-Ala-Ala-AAsn-CH=CH-CON(CH$_3$)CH$_2$-1-naphthyl	58	20
29p	Boc-Piz-Ala-Ala-AAsn-CH=CH-CON(CH$_2$-1-naphthyl)$_2$	68	5
30a	Cbz-Piz-Ala-Ala-AAsn-CH=CH-COOEt	70	0.14
30n	Cbz-Piz-Ala-Ala-AAsn-CH=CH-CON(CH$_3$)CH$_2$-1-naphthyl	70	0.35
30p	Cbz-Piz-Ala-Ala-AAsn-CH=CH-CON(CH$_2$-1-naphthyl)$_2$	50	0.37

Among the inhibitors with the Mu-Ala-Ala-AAsn peptide sequence, the ethyl ester analog (**28a**) was as effective (IC_{50} = 101 nM) as the disubstituted amide analog (**28k**) (IC_{50} = 103 nM) against SmAE. However, in the case of IrAE, replacement of the ethyl ester (IC_{50} = 0.23 nM) with the disubstituted amide analog, tetrahydroisoquinoline (IC_{50} = 65 nM), result in 300-fold decrease in potency. This is one of very few examples where the parasite AEs diverged markedly in their SAR. We conclude that even though IrAE does prefer disubstituted amides, for example, the dinaphthylmethylene analog (**28p**) (IC_{50} = 3.3 nM), it does not favor constrained amides in the P1'. The monoamide analog (**28h**) (SmAE, IC_{50} = 1450 nM; IrAE, IC_{50} >2,000 nM) was a poor inhibitor of both enzymes. There can be two reasons for this; either the alkyl spacer puts the phenyl group in an unfavorable position or the presence of a hydrogen bond donor group is not favored in the S1' position.

Next, we synthesized five inhibitors with Piz-Ala-Ala-AAsn peptide sequence. The two ester derivatives (ethyl ester, **37a** and benzyl ester, **37b**) were moderate inhibitors of both SmAE (IC_{50} = 300 nM and IC_{50} = 250 nM, respectively) and IrAE (IC_{50} = 8.5 nM and IC_{50} = 13 nM, respectively). However, the disubstituted amide derivatives (**37l**, **n**, **p**) were better against SmAE (IC_{50} = 90 nM, IC_{50} = 40 nM and IC_{50} = 70 nM). Because these compounds contain aromatic groups in their warheads, we conclude that the presence of such groups at P1' improves binding of the inhibitors due to π-stacking in either S1' or S2' of SmAE. Piz-Ala-Ala-AAsn-CH=CH-CON(CH_3)CH_2-1-naphthyl (**37n**) was the most potent inhibitor against SmAE (IC_{50} = 40 nM) among all the compounds synthesized. Probably, the methyl group interacts with a small hydrophobic pocket in the S1', whereas the naphthyl group reaches to a larger hydrophobic pocket in the S2'.

Then, we synthesized eight compounds with protecting groups on the piperazine; five of these (**29a-b, l, n, p**) contain the *tert*-butoxycarbonyl (Boc) group whereas the other three (**30a, n, p**) contain the benzyloxycarbonyl (Cbz) group. Interestingly, all the compounds with Boc-Piz-Ala-Ala-AAsn and Cbz-Piz-Ala-Ala-AAsn peptide sequences were potent inhibitors of both SmAE and IrAE. We speculate that there is a hydrophobic pocket that is interacting with hydrophobic groups (Boc and Cbz) in the P5 position. In particular, the Cbz-Piz-Ala-Ala-AAsn peptide sequence (**30a, n, p**) produced the best inhibitors of IrAE (IC_{50} = 0.14, 0.35 and 0.37 nM, respectively) herein reported. As the Boc-Piz-Ala-Ala-AAsn compounds were not as potent as those containing Cbz-Piz-Ala-Ala-AAsn, we conclude that the increased π-stacking of the aromatic residues in the S5 pocket improves inhibitor binding.

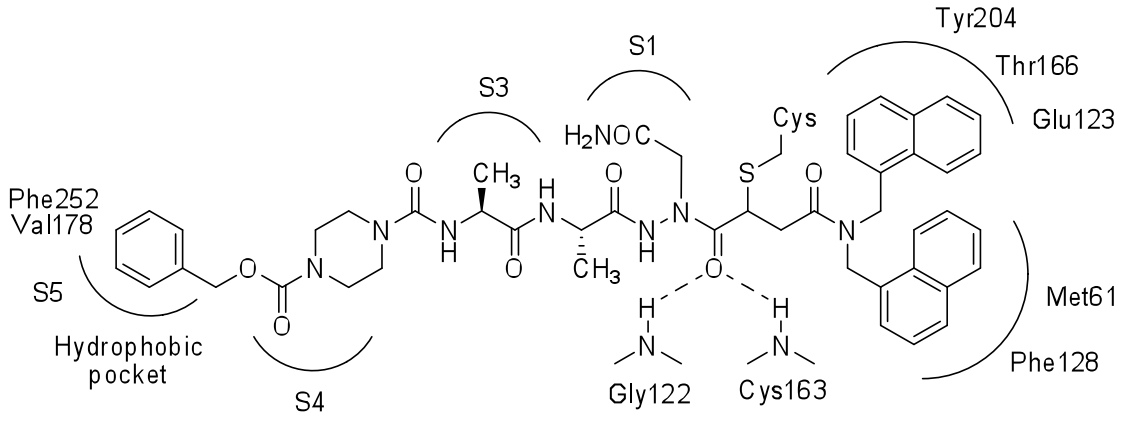


Figure 2.11. Proposed Mode of Binding of Aza-peptide Michael Acceptor to the Active Site of Asparagine Endopeptidases.

Asparagine endopeptidases belong to clan CD cysteine proteases. Clan CD cysteine proteases contains families with a protein fold or sequence motifs similar to

those found in the caspase family. Since there is no crystal structure determined for asparagine endopeptidases, we studied the crystal structures of caspases to predict the interaction sites of inhibitors with the enzyme (Figure 2.11). The crystal structures of caspase-3 in complex with aza-peptide Michael acceptors demonstrated that there are two hydrophobic pockets in the primed site. One of the hydrophobic pockets formed by Tyr 204, Thr166, and Glu123 while the other one formed by Met61 and Phe128. The P1 carbonyl group is stabilized by hydrogen bonds to the backbone amide nitrogens of Gly122 and Cys163. The most interesting discovery was the hydrophobic pocket in the S5 site of caspases. The inhibitors with Boc-Piz-Ala-Ala-AAsn and Cbz-Piz-Ala-Ala-AAsn peptide sequence were the most potent inhibitors presented here and we proposed that the protecting groups (Boc and Cbz) are interactiong with the hydrophobic pocket formed by Phe252 and Val178. Especially, the inhibitors with Cbz-Piz-Ala-Ala-AAsn peptide sequence have the lowest IC_{50} values and we proposed that the phenyl ring of the Cbz group stacks against the phenylalanine residue in the hydrophobic pocket.

To study the effect of P2 residue on inhibitor potency, we synthesized seven compounds with Cbz-Ala-AA-AAsn (AA= Val, Ile, and Phe) peptide sequence. The results are shown in Table 2.4. Compounds with valine (**31a, m, j**) or isoleucine (**32a**) residue at P2 were good to poor inhibitors whereas, those containing phenylalanine at P2 (**33e, j, k**) were extremely poor against SmAE and IrAE, confirming that both enzymes do not prefer large hydrophobic groups in the S2 position. With respect to the ethyl ester compounds **31a** and **32a** that differ only at P2, SmAE reacted with both equally, whereas inhibition of IrAE was 100-fold better with **32a**. This is a second example of divergent SAR between these enzymes and suggests that IrAE can accept larger aliphatic

hydrophobic residues at S2. Previous studies with positional scanning substrate combinatorial libraries tested against SmAE[51] and IrAE[33] demonstrated that the preferred amino acids at P2 are Ala>Val>Ile>Phe and Ile>Ala>Val>Phe, respectively. Thus, our results are in good agreement with these data.

Table 2.4. Inhibition of SmAE and IrAE by Cbz Protected Tripeptide Michael Acceptors.

	Inhibitor	SmAE IC_{50} (nM)	IrAE IC_{50} (nM)
31a	Cbz-Ala-Val-AAsn-CH=CH-COOEt	100	65
31m	Cbz-Ala-Val-AAsn-CH=CH-CONHCH$_2$-1-naphthyl	1050	350
31j	Cbz-Ala-Val-AAsn-CH=CH-CO-tetrahydroquinoline	138	13
32a	Cbz-Ala-Ile-AAsn-CH=CH-COOEt	280	0.52
33e	Cbz-Ala-Phe-AAsn-CH=CH-CONHCH$_2$-2-furyl	>2,000	1,800
33j	Cbz-Ala-Phe-AAsn-CH=CH-CO-tetrahydroquinoline	>2,000	500
33k	Cbz-Ala-Phe-AAsn-CH=CH-CO-tetrahydroisoquinoline	>2,000	500

Inhibition of SmAE and IrAE with Aza-peptidyl Epoxides. The IC_{50} values of eight aza-peptide epoxide inhibitors are listed in Table 2.5. Comparison of the three aza-peptidyl epoxide inhibitors with Pip-Ala-Ala-AAsn sequence showed that the ester analog (**34q**) and the monosubstituted amide analog (**34s**) were moderate inhibitors against SmAE with the IC_{50} values of 220 and 160 nM, respectively; however, the disubstituted amide derivative (**34r**) was best with an IC_{50} value of 72 nM. For IrAE, all compounds were moderate inhibitors but unlike SmAE, IrAE favored the

monosubstituted amide analog (IC_{50} = 8.5 nM) to the ethyl ester analog (IC_{50} = 30 nM) and the disubstituted amide analog (IC_{50} = 12 nM). These results showed that the two enzyme differs in their S1' or S2' positions slightly. Replacement of the piperidine residue in the P4 position of compound **34s** (SmAE, IC_{50} = 160 nM, IrAE, IC_{50} = 8.5 nM) with a morpholine residue in compound **35s** (IC_{50} = 60 nM) resulted in an almost three-fold increase in potency against SmAE, whereas it had almost no effect against IrAE (IC_{50} = 6 nM).

Table 2.5. Inhibition of SmAE and IrAE by Aza-Peptide Epoxides.

	Inhibitor	SmAE IC_{50} (nM)	IrAE IC_{50} (nM)
34q	Pip-Ala-Ala-AAsn-EP(*S,S*)-COOEt	220	30
34r	Pip-Ala-Ala-AAsn-EP(*S,S*)-CON(Bzl)$_2$	72	12
34s	Pip-Ala-Ala-AAsn-EP(*S,S*)-CONHCH$_2$-1-naphthyl	160	8.5
35s	Mu-Ala-Ala-AAsn-EP(*S,S*)-CONHCH$_2$-1-naphthyl	60	6
35r	Mu-Ala-Ala-AAsn-EP(*S,S*)-CON(Bzl)$_2$	90	1.7
35t	Mu-Ala-Ala-AAsn-EP(*cis*)-CON(Bzl)$_2$	N.I.	N.I.
38r	Piz-Ala-Ala-AAsn-EP(*S,S*)-CON(Bzl)$_2$	150	11
36r	Boc-Piz-Ala-Ala-AAsn-EP(*S,S*)-CON(Bzl)$_2$	80	270

To study the effect of stereochemistry on inhibitor potency, we synthesized two dibenzyl amide epoxide warheads with (*S,S*) and *cis* configurations, and coupled them to the Mu-Ala-Ala-AAsn peptidyl precursor to form compounds **35r** and **35t**, respectively. Compound **35t** (*cis*) was inactive against both enzymes, whereas the (*S,S*) compound retained potency. Accordingly, we suggest that the (*cis*) stereochemistry of the epoxide

unfavorably changes the orientation of the prime side substituents to cause a loss of inhibition. Piz-Ala-Ala-AAsn-EP(*S,S*)-CON(Bzl)$_2$ (**38r**) was a moderate inhibitor against both SmAE and IrAE with IC_{50} values of 150 and 11 nM, respectively. Replacement of the piperazine with *tert*-butoxycarbonyl piperazine (**36r**) increased the potency 2-fold against SmAE. On the other hand, the same replacement resulted in 24-fold decrease in potency against IrAE. Extension of the aza-peptides to the P5 position by introduction of Boc group resulted in increased potency in all compounds except this one example.

Inhibition of SmAE and IrAE with Aza-peptidyl substrate analog inhibitors. Finally, we synthesized five aza-peptidyl substrate analog inhibitors, however, none of these compounds inhibited SmAE or IrAE (Table 2.6).

Table 2.6. Inhibition of SmAE and IrAE by Aza-Peptide Substrate Analogs.

	Inhibitor	SmAE IC_{50} (nM)	IrAE IC_{50} (nM)
39	Mu-Ala-AAsn-$COCH_2CH_2Ph$	N.I.	N.I.
40	Pip-Ala-AAsn-CONHPh-2-OPh	N.I.	N.I.
41	Mu-Ala-AAsn-CONH-1-naphthyl	N.I.	N.I.
42	Cbz-Piz-Ala-AAsn-$CONHCH_2Ph$	N.I.	N.I.
43	Mu-Ala-Ala-AAsn-$COOCH_2Ph$	N.I.	N.I.

Second order inhibition rate constants for a selection of the aza-peptide Michael acceptors and epoxides are presented in Table 2.7. We compared the rates of our compounds with the gold standard inhibitor Cbz-Ala-Ala-AAsn-CH=CH-COOEt[55] (k_{inact}/K_{iapp}=160,000 $M^{-1}s^{-1}$ for SmAE and 640,000 $M^{-1}s^{-1}$ for IrAE). In the case of

Table 2.7. Comparison of Inhibition of AEs by Aza-peptide Michael Acceptors and Epoxides.

Inhibitor	SmAE k_{inact}/K_{iapp} $(M^{-1}s^{-1})x10^{-6}$	IrAE k_{inact}/K_{iapp} $(M^{-1}s^{-1})x10^{-6}$
Cbz-Ala-Ala-AAsn-CH=CH-COOEt[55]	0.16	0.64
Piz-Ala-Ala-AAsn-CH=CH-COOBzl	0.046	0.17
Boc-Piz-Ala-Ala-AAsn-CH=CH-COOBzl	0.023	0.67
Piz-Ala-Ala-AAsn-EP(*S,S*)-CON$(Bzl)_2$	0.03	0.10
Pip-Ala-Ala-AAsn-EP(*S,S*)-CON$(Bzl)_2$	0.037	0.45
Boc-Piz-Ala-Ala-AAsn-EP(*S,S*)-CON$(Bzl)_2$	0.01	0.39
Boc-Piz-Ala-Ala-AAsn-CH=CH-CON$(Bzl)_2$	0.04	0.35
Mu-Ala-Ala-AAsn-CH=CH-CON$(CH_2$-1-naph$)_2$	0.4	1.98
Pip-Ala-Ala-AAsn-CH=CH-CON$(CH_2$-1-naph$)_2$	0.27	0.77
Cbz-Piz-Ala-Ala-AAsn-CH=CH-CON$(CH_3)CH_2$-1-naph	0.084	1.79
Piz-Ala-Ala-AAsn-CH=CH-CON$(CH_3)CH_2$-1-naph	0.16	0.22

SmAE, two compounds (**28p**, 400,000 $M^{-1}s^{-1}$ and **27p**, 270,000 $M^{-1}s^{-1}$) have higher rates than Cbz-Ala-Ala-AAsn-CH=CH-COOEt and one compound (**37n**, 160,000 $M^{-1}s^{-1}$) has the same rate. All three compounds have Michael acceptors as a warhead and disubstituted amides with aromatic residues. In general, aza-peptide Michael acceptors inhibit SmAE more rapidly than epoxide analogs. For IrAE, three compounds (**29b**, 670,000 $M^{-1}s^{-1}$ and **30n**, 1,790,000 $M^{-1}s^{-1}$ and **28p**, 1,980,000 $M^{-1}s^{-1}$ have higher rates than Cbz-Ala-Ala-AAsn-CH=CH-COOEt. Again, all compounds were aza-peptide Michael acceptors with aromatic residues in the P1' position.

Mechanism of Inhibition. Aza-peptide Michael acceptors and epoxides are irreversible inhibitors of Clan CD cysteine proteases.[55]

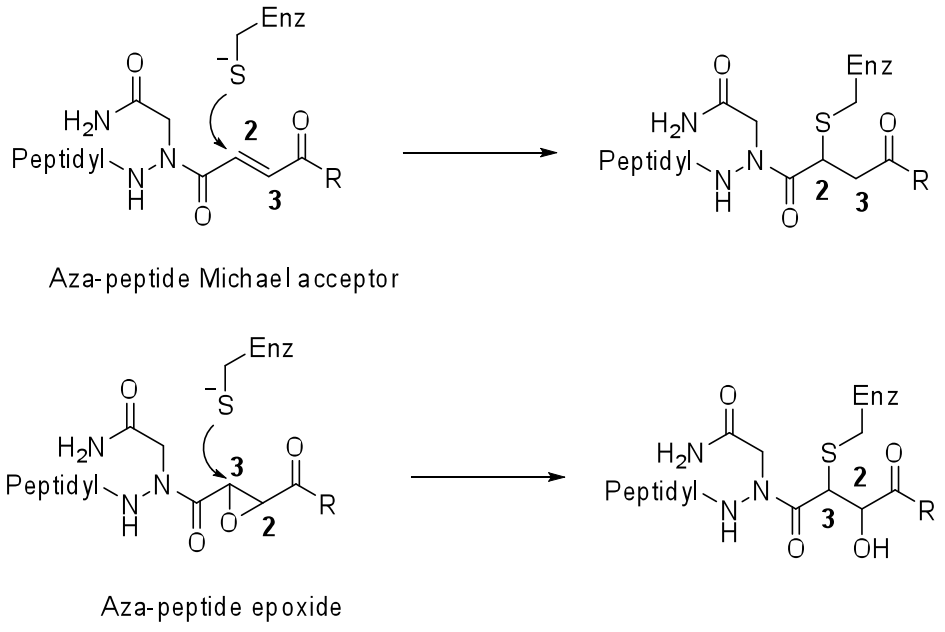


Figure 2.12. Mechanism of Inhibition of Cysteine Proteases by Michael Acceptor and Epoxide Inhibitors.

The mechanism of inhibition involves nucleophilic attack by the catalytic cysteine residue on the Michael acceptor double bond or epoxide forming a covalent bond and irreversibly inhibiting the enzyme.[63] Theoretically, either C2 or C3 carbon of the Michael acceptor and epoxide moiety could be attacked by the active site cysteine residue. X-ray crystal structures of aza-peptide Michael acceptor inhibitors bound to caspase-3 and caspase-8 have shown that the nucleophilic attack of the active site cysteine residue occurs at the C2 carbon of the double bond.[64] On the other hand, crystal structures of aza-peptide epoxide inhibitors bound to caspase-3 have shown that the site of attack is at the C3 carbon of the epoxide moiety.[65] These structures demonstrate that the site of attack is the same in both warheads, i.e., the carbon closest to the aza-peptide nitrogen atom (see Figure 2.12 for different numbering of Michael acceptor and epoxide moieties). In view of these data, we suggest that, for AEs, the covalent bond forms at the C2 carbon of the Michael acceptors and at the C3 carbon of the epoxides.

CONCLUSION

We have demonstrated that aza-peptide Michael acceptors and epoxides with the general structure Boc-AAsn-*trans*-CH=CHCOR, YCO-Ala-AAsn-*trans*-CH=CHCOR, YCO-Ala-Ala-AAsn-*trans*-CH=CHCOR and YCO-Ala-Ala-AAsn-EP-COR are inhibitors of SmAE and IrAE. We have shown that tripeptides (YCO-Ala-Ala-AAsn-*trans*-CH=CHCOR) are favored over dipeptides (YCO-Ala-AAsn-*trans*-CH=CHCOR) that, in turn, are favored over monopeptides (Boc-AAsn-*trans*-CH=CHCOR).

This research discovered the importance of the S5 subsite in the active sites of both SmAE and IrAE. Extension of the inhibitor structure to P5 with the introduction of Cbz and Boc groups resulted in increased potenty. For some of these compounds, we obtained IC_{50} values as low as 0.14 nM and second order inhibiton rate constants are the fastest yet discovered. We have shown the possible interactions of the aza-peptide Michael acceptor inhibitor with the enzyme by studing the crystal structures of caspase-3. We proposed for the first time that the inhibitors which are extended to the P5 position are interacting with a hydrophobic pocket in the S5 site of SmAE and IrAE. Both enzymes prefer disubstituted amides to monosubstituted amides in the P1' position and we found that potency of the inhibitors increased as we increased the hydrophobicity in the P1'.

Aza-peptide Michael acceptor inhibitors were more potent than aza-peptide epoxide inhibitors. We have also determined that the stereochemistry of the epoxide warhead is important; (*S,S*) is favored over (*cis*). In addition, both enzymes prefer small hydrophobic amino acids like valine and isoleucine in the P2 position, whereas large hydrophobic amino acids such as phenylalanine are not tolerated.

There has been no success in the determination of a crystal structure of the asparaginyl endopeptidases. Therefore potent and selective irreversible inhibitors would help to determine the structural and enzymatic specificities of the active site of asparagine endopeptidases. There is also an urgent need for the development of new inhibitors of SmAE and IrAE due to increasing resistance of the parasites to the current drugs. We have synthesized the most potent inhibitors against same and IrAE reported in the literature. Given the central functions of these enzymes in nutrient acquisition by

both parasites, the data and SAR presented here may facilitate the design of selective anti-parasitic drugs.

Significance

This research increased our understanding of the active sites of both enzymes and the interactions of the inhibitors with these enzymes. The data from this research will facilitate the development of therapeutically useful compounds which will be beneficial to millions of people who suffer from these parasitic diseases.

EXPERIMENTAL

Material and Methods. Materials were obtained from Acros, Bachem Bioscience Inc., or Sigma Aldrich and used without further purification. The purity of each compound was confirmed by TLC, ^{1}H NMR, MS, and elemental analysis. Chemical shifts are reported in ppm relative to an internal standard (trimethylsilane). TLC was performed on Sorbent Technologies (250 μm) silica gel plates. The ^{1}H NMR spectra were obtained on a Varian Mercury 400 MHz spectrometer. Electrospray ionization (ESI), fast-atom-bombardment (FAB) and high-resolution mass spectrometry were obtained using Micromass Quattro LC and VG Analytical 70-SE instruments. Elemental analysis was carried out by Atlantic Microlab Inc., Norcross, GA. The synthetic aza-peptide inhibitors were tested against *S. mansoni* and *I. ricinus* AEs by Dr. Caffrey in the laboratory of Jim McKerrow at the University of California, San Francisco.

Determination of IC_{50} values and second-order inhibition rates for Asparagine Endopeptidases. IC_{50} values were determined exactly as previously described.[49] For second-order rate inhibitions, the substrate Cbz-Ala-Ala-Asn-AMC and inhibitors were prepared as 10 and 20 mM stock solutions, respectively, in DMSO. Assays were performed at 25°C and incorporated either activated recombinant, *I. ricinus* asparaginyl endopeptidase[33] or activated recombinant *S. mansoni* asparaginyl endopeptidase.[66] An aliquot of (100 µl) *S. mansoni* AE in assay buffer (0.1M citrate-phosphate buffer at pH 6.8 containing 4 mM DTT) was added to 100 µl inhibitor in assay buffer with 20 µM substrate and inhibitor present at 2, 1, 0.8, 0.6, 0.4, 0.2 and 0.1 µM (or DMSO alone) added as a 1 µl aliquot. The progress of inhibition was followed for 2 to 5 minutes, while the uninhibited activity was linear. An aliquot of (100 µl) *I. ricinus* AE in assay buffer (0.1M citrate-phosphate buffer at pH 6.8 containing 4 mM DTT with 0.1 M NaCl) was added to inhibitor, in 100µl assay buffer with inhibitor present at 1, 0.8, 0.5, 0.25, 0.125, 0.0625, 0.03125 µM and DMSO alone, as above. Release of free AMC was measured at emission and excitation wavelengths of 355 and 460 nm, respectively, in a Molecular Devices Flex-Station fluorometer. Measurements were taken every 1.52 s. The rate of inhibition (k_{obs}) was determined at each inhibitor concentration according to $[P] = v_o / k_{obs} \times [1- \exp(- k_{obs} \times t)]$, where [P] is the concentration of product formed over time t and v_o the initial rate, using non-linear regression analysis (GRAPHPAD PRISM 3.0a). If the k_{obs} versus inhibitor concentration could reliably fit a two-step irreversible mechanism ($r^2 > 0.9$), the inhibition constant K_i and the inactivation constant k_{inact} were determined by nonlinear regression analysis according to $k_{obs} = k_{inact} \times [I]_o / ([I]_o + K^*_I \times (1+ [S]_o / K_m))$ where $[I]_o$ and $[S]_o$ are the concentrations of inhibitor and substrate,

respectively, and $K^*_I = K_i \times (1 = [S]_o / K_m)$. For those inhibitors with which k_{obs} was linear with increasing concentrations of inhibitor, linear regression analysis was used to obtain the association constant k_{ass} using $k_{obs} = k_{ass} \times [I]_o / (1 + [S]_o/K_m)$. Multiple determinations at each inhibitor concentration were performed.

General Procedure for the Synthesis of Acyl Alanine Methyl Ester (YCO-Ala-OCH_3). Alanine methyl ester isocyanate, which is synthesized from alanine hydrochloride salt according to the procedure by Nowick et al.,[60] is dissolved in CH_2Cl_2 and cooled down to 0 °C. Y-H groups, which are commercially available except the benzyloxycarbonyl piperazine and *tert*-butoxycarbonyl piperazine are added to the stirred solution of alanine methyl ester isocyanate. Reaction mixture was stirred vigorously for 16 h at room temperature. Solvent was removed under vacuum Purification on a silica gel column with the proper eluent gave the product with yields of 72-93%.

tert-Butoxycarbonyl piperazine (Boc-Piz). A solution of di-tert-butyl dicarbonate (1 eq) in dichloromethane was added slowly to a solution of piperazine (2 eq) in dichloromethane at 0 °C. The reaction mixture was stirred at room temperature overnight. The dichloromethane solution was washed with saturated $NaHCO_3$ and water. Diethyl ether was added, and the organic phase was washed with water. The organic phase was dried with anhydrous Na_2SO_4 and evaporated. Purification was by column chromatography gave the product with 57% yield. ^{1}H NMR ($CDCl_3$): 1.42 (s, 9H, Boc), 2.00 (br s, 1H), 2.78 (m, 4H, piperazine), 3.36 (m, 4H, piperazine). MS (ESI) *m/z* 187.1 $[(M + 1)^+]$.

Benzyloxycarbonyl piperazine (Cbz-Piz). Monoprotection of piperazine was done with benzyl chloroformate (1 eq) and Et_3N (1 eq) in dichloromethane. The solvent

was evaporated and the crude product was purified by column chromatography to obtain the product with 55% yield. ^{1}H NMR (DMSO-d_6): 2.33 (s, 1H, NH), 2.62 (m, 4H, piperazine), 3.30-3.36 (m, 4H, piperazine), 5.00 (s, 2H, Cbz), 7.15-7.22 (m, 5H, Ph). MS (ESI) *m/z* 221.0 [$(M + 1)^+$].

The piperidine derivative Pip-Ala-OCH_3 was prepared by adding piperidine to alanine isocyanate and purified by column chromatography on silica gel using 2:18:5 MeOH:CH_2Cl_2:EtOAc as the eluent; white solid, yield 83%. ^{1}H NMR ($CDCl_3$-d): 1.32 (d, 3H, Ala-CH_3), 1.48 (m, 6H, piperidine), 3.27 (m, 4H, piperidine), 3.67 (s, 3H, OCH_3), 4.41 (m, 1H, α-H), 5.05 (d, 1H, NH). MS (ESI) *m/z* 215.0 [$(M + 1)^+$].

The morpholine derivative Mu-Ala-OCH_3 was prepared by adding morpholine to alanine isocyanate and purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent; yellowish solid, yield 93%. ^{1}H NMR ($CDCl_3$-d): 1.29 (d, 3H, Ala-CH_3), 3.28 (m, 4H, morpholine), 3.58 (m, 4H, morpholine), 3.64 (s, 3H, OCH_3), 4.38 (m, 1H, α-H), 5.25 (s, 1H, NH). MS (ESI) *m/z* 217.0 [$(M + 1)^+$].

The tert-butoxycarbonyl piperazine derivative Boc-Piz-Ala-OCH_3 was prepared by adding tert-butoxycarbonyl piperazine to alanine isocyanate and purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent; white solid, yield 80%. ^{1}H NMR ($CDCl_3$-d): 1.37 (d, 3H, Ala-CH_3), 1.44 (s, 9H, Boc), 3.34-3.44 (m, 8H, piperazine), 3.72 (s, 3H, OCH_3), 4.47 (m, 1H, α-H), 5.08 (s, 1H, NH). MS (ESI) *m/z* 316.2 [$(M + 1)^+$].

The benzyloxycarbonyl piperazine derivative Cbz-Piz-Ala-OCH_3 was prepared by adding benzyloxycarbonyl piperazine to alanine isocyanate and was purified by column chromatography on silica gel using 2:18:5 MeOH:CH_2Cl_2:EtOAc as the eluent; white

solid, yield 72%. ^{1}H NMR ($CDCl_3$-d): 1.31 (d, 3H, Ala-CH_3), 3.34-3.36 (m, 4H, piperazine), 3.44-3.47 (m, 4H, piperazine), 3.65 (s, 3H, OCH_3), 4.39 (m, 1H, α-H), 5.07 (s, 2H, Cbz), 5.41 (d, 1H, NH), 7.23-7.29 (m, 5H, Ph). MS (ESI) *m/z* 350.2 [$(M + 1)^+$].

General Procedure for the Synthesis of Acyl Dipeptide Methyl Ester (YCO-Ala-Ala-OCH_3). The methyl ester group of Y-Ala-OCH_3 was hydrolyzed in MeOH using 1 M aqueous NaOH (1.1 eq) under standard deblocking conditions. Y-Ala-OH is then coupled to alanine methyl ester hydrochloride ($HCl{\cdot}NH_2$-Ala-OCH_3) using DCC/HOBt coupling. To a stirred solution of the Y-Ala-OH (1 eq) in DMF at -15 °C, HOBt (1.5 eq) was added. Hydrochloride salt of the alanine methyl ester was pretreated with NMM (1.5 eq) at -15 °C DMF prior to addition. DCC (1.5 eq) was added to the solution and reaction mixture was allowed to react for 16 h at room temperature. The DMF was evaporated, and the residue was redissolved in EtOAc. The organic layer was washed with 2% citric acid, saturated $NaHCO_3$, saturated NaCl, dried over $MgSO_4$, and concentrated. Purification on a silica gel column with the proper eluent gave the product with yields of 60-89%.

Pip-Ala-OH was obtained by hydrolysis of Pip-Ala-OCH_3 in MeOH using 1 M aqueous NaOH (1.1 eq); white solid, yield 85%. ^{1}H NMR (DMSO-d_6): 1.23 (d, 3H, Ala-CH_3), 1.39 (m, 4H, piperidine), 1.49, (d, 2H, piperidine), 3.24 (s, 4H, piperidine), 4.03 (m, 1H, α-H), 6.50 (d, 1H, NH).

After coupling Pip-Ala-OH with $HCl{\cdot}NH_2$-Ala-OCH_3, Pip-Ala-Ala-OCH_3 was purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent; white solid, yield 78%. ^{1}H NMR ($CDCl_3$-d): 1.37-1.40 (m, 6H, 2 x Ala-CH_3), 1.54-1.59

(m, 6H, piperidine), 3.31-3.34 (m, 4H, piperidine) 3.73 (s, 3H, OCH_3), 4.45-4.53 (m, 2H, 2 x α-H), 5.21 (s, 1H, NH), 7.12 (s, 1H, NH). MS (FAB) *m/z* 286.0 [$(M + 1)^+$].

Mu-Ala-OH was obtained by hydrolysis of Pip-Ala-OCH_3 in MeOH using 1 M aqueous NaOH (1.1 eq); white solid, yield 89%. ^{1}H NMR (DMSO-d_6): 1.24 (d, 3H, Ala-CH_3), 3.26 (t, 4H, morpholine) 3.51 (t, 4H, morpholine), 4.05 (m, 1H, α-H), 6.65 (d, 1H, NH), 12.21 (br s, 1H, COO*H*). MS (FAB) *m/z* 202.9 [$(M + 1)^+$].

After coupling Mu-Ala-OH with HCl·NH_2-Ala-OCH_3, Mu-Ala-Ala-OCH_3 was purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent; white solid, yield 60%. ^{1}H NMR ($CDCl_3$-d): 1.37 (d, 3H, Ala-CH_3), 1.39 (d, 3H, Ala-CH_3), 3.34-3.36 (m, 4H, morpholine), 3.65-3.67 (m, 4H, morpholine) 3.73 (s, 3H, OCH_3), 4.45-4.53 (m, 2H, 2 x α-H), 5.32 (s, 1H, NH), 7.02 (d, 1H, NH). MS (FAB) *m/z* 288.2 [$(M + 1)^+$].

Boc-Piz-Ala-OH was obtained by hydrolysis of Boc-Piz-Ala-OCH_3 in MeOH using 1 M aqueous NaOH (1.1 eq); white solid, yield 94%. ^{1}H NMR (DMSO-d_6): 1.24 (d, 3H, Ala-CH_3), 1.38 (s, 9H, Boc), 3.26 (s, 8H, piperazine), 4.04 (m, 1H, α-H), 6.69 (d, 1H, NH).

After coupling Boc-Piz-Ala-OH with HCl·NH_2-Ala-OCH_3, Boc-Piz-Ala-Ala-OCH_3 was purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent; white solid, yield 85%. ^{1}H NMR ($CDCl_3$-d): 1.36-1.39 (m, 6H, 2 x Ala-CH_3), 1.44 (s, 9H, Boc), 3.34-3.39 (m, 8H, piperazine), 3.72 (s, 3H, OCH_3), 4.44-4.54 (m, 2H, 2 x α-H), 5.38 (d, 1H, NH), 7.09 (d, 1H, NH).

Cbz-Piz-Ala-OH was obtained by hydrolysis of Cbz-Piz-Ala-OCH_3 in MeOH using 1 M aqueous NaOH (1.1 eq); white solid, yield 87%. ^{1}H NMR (DMSO-d_6): 1.25

(d, 3H, Ala-CH_3), 3.32-3.34 (m, 8H, piperazine), 4.06 (m, 1H, α-H), 5.07 (s, 2H, Cbz), 6.72 (d, 1H, NH), 7.28-7.37 (m, 5H, Ph).

After coupling Cbz-Piz-Ala-OH with HCl·NH_2-Ala-OCH_3, Cbz-Piz-Ala-Ala-OCH_3 was purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent; white solid, yield 89%. ^{1}H NMR ($CDCl_3$-d): 1.38 (m, 6H, Ala-CH_3), 3.38 (m, 4H, piperazine), 3.50 (m, 4H, piperazine), 3.72 (s, 3H, OCH_3), 4.46 (m, 1H, α-H), 5.12 (s, 2H, Cbz), 7.34 (m, 5H, Ph).

General Procedure for the Synthesis of Cbz Protected Dipeptide Methyl Esters (Cbz-Ala-Val(Ile, Phe)-OCH_3). Cbz-Ala-OH is coupled to valine methyl ester (isoleucine methyl ester, or phenylalanine methyl ester) using DCC and HOBt coupling. To a stirred solution of the Cbz-Ala-OH (1 eq) in DMF at -15 °C, HOBt (1.5 eq) was added. Hydrochloride salt of the valine methyl ester was pretreated with NMM (1.5 eq) at -15 °C DMF prior to addition. DCC (1.5 eq) was added to the solution and reaction mixture was allowed to react for 16 h at room temperature. The DMF was evaporated, and the residue was redissolved in EtOAc. The organic layer was washed with 2% citric acid, saturated $NaHCO_3$, saturated NaCl, dried over $MgSO_4$, and concentrated.

After coupling Cbz-Ala-OH with HCl·NH_2-Val-OCH_3, Cbz-Ala-Val-OCH_3 was purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent; white solid, yield 85%. ^{1}H NMR ($CDCl_3$-d): 0.88 (m, 6H, 2 x Val-CH_3), 1.39 (d, 3H, Ala-CH_3), 2.16 (m, 1H, CH), 3.74 (s, 3H, OCH_3), 4,27 (m, 1H, α-H), 4.52 (m, 1H, α-H), 5.12 (s, 2H, Cbz), 5.36 (d, 1H, NH), 6.54 (d, 1H, NH), 7.36–7.32 (m, 5H, Ph). MS (ESI) *m/z* 337.1 [$(M + 1)^+$].

After coupling Cbz-Ala-OH with $HCl \cdot NH_2$-Ile-OCH_3, Cbz-Ala-Ile-OCH_3 was purified by column chromatography on silica gel using 1:1 EtOAc:Hexanes as the eluent; white solid, yield 89%. ^{1}H NMR ($CDCl_3$-d): 0.85-0.89 (m, 6H, 2 x Ile-CH_3), 1.08-1.19 (m, 2H, CH_2), 1.29-1.43 (m, 3H, Ala-CH_3), 1.86 (m, 1H, CH), 3.70 (s, 3H, OCH_3), 4.34 (q, 1H, α-H), 4.55 (q, 1H, α-H), 5.09 (m, 2H, Cbz), 5.63 (d, 1H, NH), 6.82 (d, 1H, NH), 7.26-7.36 (m, 5H, Ph).

After coupling Cbz-Ala-OH with $HCl \cdot NH_2$-Phe-OCH_3, Cbz-Ala-Phe-OCH_3 was purified by column chromatography on silica gel using 2:1 EtOAc:hexane as the eluent; white solid, yield 86%. ^{1}H NMR ($CDCl_3$-d): 1.32 (d, 3H, Ala-CH_3), 3.03-3.16 (m, 2H, *CH_2*Ph), 3.70 (s, 3H, OCH_3), 4.25 (q, 1H, α-H), 4.85 (q, 1H, α-H), 5.08 (m, 2H, Cbz), 5.38 (d, 1H, NH), 7.08 (d, 1H, NH), 7.21-7.34 (m, 10 H, 2 x Ph). MS (ESI) *m/z* 385.0 $[(M + 1)^+]$.

Peptidyl Hydrazides. Anhydrous hydrazine (10 equiv) was added to a stirred solution of a peptidyl methyl ester (1 equiv) in MeOH and the reaction mixture was stirred vigorously for 16 h at room temperature. Excess hydrazine and solvent were removed under vacuum and the residue was washed with ether several times to give the peptidyl hydrazide as a white solid. Compounds were purified further on silica gel column using the eluent 10% MeOH/CH_2Cl_2 when needed.

Pip-Ala-NH-NH_2, white solid, yield 97%. ^{1}H NMR (DMSO-d_6): 1.15 (d, 3H, Ala-CH_3), 1.36-1.41 (m, 4H, piperidine), 1.46-1.49 (m, 2H, piperidine), 3.22-3.25 (m, 4H, piperidine), 4.06-4.12 (m, 3H, α-H and NH_2), 6.28 (d, 1H, NH), 8.96 (s, 1H, NH). MS (ESI) *m/z* 215.0 $[(M + 1)^+]$.

Mu-Ala-NH-NH_2, white solid, yield 97%. ^{1}H NMR (DMSO-d_6): 1.16 (d, 3H, Ala-CH_3), 3.23-3.26 (m, 4H, morpholine), 3.49-3.52 (m, 4H, morpholine), 4.07-4.14 (m, 3H, α-H and NH_2), 6.45 (d, 1H, NH), 8.97 (s, 1H, NH). MS (ESI) *m/z* 217.0 [$(M + 1)^+$].

Cbz-Piz-Ala-NH-NH_2, white solid, yield 98%. ^{1}H NMR (DMSO-d_6): 1.16 (d, 3H, AlaCH_3), 3.30-3.34 (m, 8H, piperazine), 4.11 (m, 3H, α-H and NH_2), 5.07 (s, 2H, Cbz), 6.51 (d, 1H, NH), 7.34 (m, 5H, Ph), 8.95 (s, 1H, NH).

Pip-Ala-Ala-NH-NH_2, white solid, yield 91%. ^{1}H NMR (DMSO-d_6): 1.15-1.18 (m, 6H, 2 x Ala-CH_3), 1.38-1.40 (m, 4H, piperidine), 1.48-1.52 (m, 2H, piperidine), 3.25-3.27 (m, 4H, piperidine), 4.06 (m, 1H, α-H), 4.17-4.21 (m, 3H, α-H and NH_2), 6.40 (d, 1H, NH), 7.71 (d, 1H, NH), 9.01 (s, 1H, NH). MS (ESI) *m/z* 286.0 [$(M + 1)^+$].

Mu-Ala-Ala-NH-NH_2, white solid, yield 92%. ^{1}H NMR (DMSO-d_6): 1.15-1.18 (m, 6H, 2 x Ala-CH_3), 3.25-3.28 (m, 4H, morpholine), 3.51-3.53 (m, 4H, morpholine), 4.09 (q, 1H, α-H), 4.16-4.23 (m, 3H, α-H and NH_2), 6.53 (d, 1H, NH), 7.77 (d, 1H, NH), 8.99 (s, 1H, NH).

Boc-Piz-Ala-Ala-NH-NH_2, white solid, yield 66%. ^{1}H NMR (DMSO-d_6): 1.15-1.19 (m, 6H, 2 x Ala-CH_3), 1.38 (s, 9H, boc), 3.27 (s, 8H, piperazine), 4.08 (q, 1H, α-H), 4.18-4.19 (m, 3H, α-H and NH_2), 6.56 (d, 1H, NH), 7.76 (d, 1H, NH), 8.99 (s, 1H, NH).

Cbz-Piz-Ala-Ala-NH-NH_2, white solid, yield 98%. ^{1}H NMR (DMSO-d_6): 1.15-1.18 (m, 6H, 2 x Ala-CH_3), 3.45 (s, 8H, piperazine), 4.03-4.08 (m, 1H, α-H), 4.15-4.19 (m, 3H, α-H and NH_2), 5.06 (s, 2H, Cbz), 6.58 (d, 1H, NH), 7.29-7.35 (m, 5H, Ph), 7.78 (d, 1H, NH), 9.00 (s, 1H, NH).

Cbz-Ala-Val-NH-NH_2, white solid, yield 98%. ^{1}H NMR (DMSO-d_6): 0.78-0.81 (m, 6H, 2 x Val-CH_3), 1.15 (d, 3H, Ala-CH_3), 1.82 (m, 1H, Val-CH), 4.02-4.11 (m, 2H, 2

x α-H), 4.23 (s, 2H, NH_2), 4.99 (s, 2H, Cbz), 7.29-7.34 (m, 5H, Ph),7.46 (d, 1H, NH), 7.68 (d, 1H, NH), 9.15 (s, 1H, NH).

Cbz-Ala-Ile-NH-NH_2, white solid, yield 98%. ^{1}H NMR (DMSO-d_6): 0.71-0.84 (m, 6H, 2 x Ala-CH_3), 0.97-1.05 (m, 1H, CH), 1.15 (d, 3H, CH_3), 1.38-1.44 (m, 1H, CH), 1.61-1.63 (m, 1H, CH), 4.05-4.13 (m, 2H, 2 x α-H), 4.23 (s, 2H, NH_2), 4.99 (m, 2H, Cbz), 7.29-7.36 (m, 5H, Ph),7.45 (d, 1H, NH), 7.70 (d, 1H, NH), 9.16 (s, 1H, NH).

Cbz-Ala-Phe-NH-NH_2, white solid, yield 97%. ^{1}H NMR (DMSO-d_6): 1.08 (d, 3H, Ala-CH_3), 2.78-2.97 (m, 2H, *CH_2*Ph), 3.99 (q, 1H, α-H), 4.20 (br s, 2H, NH_2), 4.42 (q, 1H, α-H), 4.99 (m, 2H, Cbz), 7.14-7.39 (m, 11 H, 2 x Ph and NH), 7.92 (d, 1H, NH), 9.14 (s, 1H, NH). MS (ESI) *m/z* 385.2 [$(M + 1)^+$].

General Procedure for the Amidation of Peptidyl-NH-NH-CH_2COOEt. To a stirred solution of a peptidyl hydrazide (1 equiv) in DMF, NMM (1.1 equiv) and *tert*-butyl bromoacetate (1.1 equiv) were added dropwise at -15 °C and the mixture was allowed to react for 36 h at room temperature. The solvent was removed under vacuum. Purification on a silica gel column with the proper eluent gave the products with yields of 20-90%.

Boc-NH-NH-CH_2COOEt was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent; white solid, yield 90%. ^{1}H NMR (DMSO-d_6): 1.26 (t, 3H, NHCH$_2$COCH$_2$*CH_3*), 1.45 (m, 9H, Boc), 4.11 (s, 2H, N*CH_2*CO), 4.19 (q, 2H, NHCH$_2$CO*CH_2*CH$_3$). MS (ESI) *m/z* 219.2 [$(M + 1)^+$].

Pip-Ala-NH-NH-CH_2COOEt was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent; yellow oil, yield 26%. ^{1}H NMR (DMSO-d_6): 0.78-0.84 (m, 3H, NHCH$_2$COCH$_2$*CH_3*), 1.13-1.19 (m, 3H, Ala-CH_3), 1.38-1.41 (m, 4H,

piperidine), 1.47-1.50 (m, 2H, piperidine), 3.23-3.25 (m, 4H, piperidine), 3.45 (d, 2H, NH*CH*$_2$COCH$_2$CH$_3$), 4.03-4.09 (m, 3H, α-H and NHCH$_2$CO*CH*$_2$CH$_3$), 5.09 (q, 1H, NH), 6.32 (d, 1H, NH), 9.16 (d, 1H, NH). MS (ESI) *m/z* 301.0 [(M + 1)$^+$].

Mu-Ala-NH-NH-CH$_2$COOEt was purified by column chromatography on silica gel using 15% MeOH/CH$_2$Cl$_2$ as the eluent; yellow oil, yield 41%. MS (ESI) *m/z* 303.0 [(M + 1)$^+$]. ^{1}H NMR (DMSO-d$_6$): 0.78-0.84 (m, 3H, NHCH$_2$COCH$_2$*CH*$_3$), 1.14-1.18 (m, 3H, Ala-CH$_3$), 3.20-3.29 (m, 4H, morpholine), 3.45 (d, 2H, NH*CH*$_2$COCH$_2$CH$_3$), 3.49-3.55 (m, 4H, *CH*$_2$O*CH*$_2$ morpholine), 4.03-4.10 (m, 3H, α-H and NHCH$_2$CO*CH*$_2$CH$_3$), 5.10 (d, 1H, NH), 6.48 (d, 1H, NH), 9.23 (d, 1H, NH).

Cbz-Piz-Ala-NH-NH-CH$_2$COOEt was purified by column chromatography on silica gel using 15% MeOH/CH$_2$Cl$_2$ as the eluent; white solid, yield 44%. ^{1}H NMR (DMSO-d$_6$): 1.14-1.18 (m, 6H, Ala-CH$_3$ and NHCH$_2$COCH$_2$*CH*$_3$), 3.30-3.33 (m, 8H, piperazine) 3.45 (d, 2H, NH*CH*$_2$COCH$_2$CH$_3$), 4.03-4.10 (m, 3H, α-H and NHCH$_2$CO*CH*$_2$CH$_3$), 5.07 (s, 2H, Cbz), 6.55 (d, 1H, NH), 7.29-7.36 (m, 5H, Ph), 9.23 (d, 1H, NH). MS (ESI) *m/z* 436.3 [(M + 1)$^+$].

Pip-Ala-Ala-NH-NH-CH$_2$COOEt was purified by column chromatography on silica gel using 15% MeOH/CH$_2$Cl$_2$ as the eluent; white solid, yield 20%. ^{1}H NMR (DMSO-d$_6$): 1.13-1.18 (m, 9H, 2 x Ala-CH$_3$ and NHCH$_2$COCH$_2$*CH*$_3$), 1.37-1.40 (m, 4H, piperidine), 1.48-1.52 (m, 2H, piperidine), 3.25 (t, 4H, piperidine), 3.44 (d, 2H, NH*CH*$_2$COCH$_2$CH$_3$), 4.04-4.09 (m, 3H, α-H and NHCH$_2$CO*CH*$_2$CH$_3$), 4.15 (m, 1H, α-H), 5.15 (m, 1H, NH), 6.40 (d, 1H, NH), 7.74 (d, 1H, NH), 9.32 (d, 1H, NH).

Mu-Ala-Ala-NH-NH-CH$_2$COOEt was purified by column chromatography on silica gel using 15% MeOH/CH$_2$Cl$_2$ as the eluent; white solid, yield 30%. ^{1}H NMR

(DMSO-d_6): 0.78-0.84 (m, 6H, 2 x CH_3), 1.14-1.18 (m, 6H, 2 x CH_3), 3.25-3.32 (m, 4H, morpholine), 3.44 (d, 2H, NH*CH_2*$COCH_2CH_3$), 3.50-3.53 (m, 4H, morpholine), 4.04-4.09 (m, 3H, α-H and $NHCH_2CO$*CH_2*CH_3), 4.18 (q, 1H, α-H), 5.15 (m, 1H, NH), 6.52 (d, 1H, NH), 7.81 (d, 1H, NH), 9.29 (d, 1H, NH).

Boc-Piz-Ala-Ala-NH-NH-CH_2COOEt was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent; white solid, yield 50%. ^{1}H NMR (DMSO-d_6): 1.26 (t, 3H, $NHCH_2COCH_2$*CH_3*), 1.34-1.37 (m, 6H, 2 x Ala-CH_3), 1.45 (s, 9H, Boc), 3.37-3.42 (m, 10H, piperazine and NH*CH_2*$COCH_2CH_3$), 4.15-4.22 (m, 2H, $NHCH_2CO$*CH_2*CH_3), 4.39 (m, 1H, α-H), 4.46 (m, 1H, α-H) and $NHCH_2CO$*CH_2*CH_3), 5.54 (d, 1H, NH), 7.35 (d, 1H, NH), 8.89 (s, 1H, NH).

Cbz-Piz-Ala-Ala-NH-NH-CH_2COOEt was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent; white solid, yield 57%. ^{1}H NMR (DMSO-d_6): 1.12-1.18 (m, 9H, 2 x Ala-CH_3 and $NHCH_2COCH_2$*CH_3*), 3.32-3.34 (m, 8H, piperazine) 3.44 (d, 2H, NH*CH_2*$COCH_2CH_3$), 4.03-4.10 (m, 4H, 2 x α-H and $NHCH_2CO$*CH_2*CH_3), 5.04 (s, 2H, Cbz), 6.59 (d, 1H, NH), 7.29-7.35 (m, 5H, Ph), 7.81 (d, 1H, NH), 9.29 (d, 1H, NH).

Cbz-Ala-Val-NH-NH-CH_2COOEt was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent; white solid, yield 64%. ^{1}H NMR (DMSO-d_6): 0.78-0.84 (m, 6H, 2 x CH_3), 1.14-1.18 (m, 6H, 2 x CH_3), 1.84 (m, 1H, CH) 3.48 (d, 2H, NH*CH_2*$COCH_2CH_3$), 4.02-4.08 (m, 4H, 2 x α-H and $NHCH_2CO$*CH_2*CH_3), 4.99 (m, 2H, Cbz), 7.28-7.34 (m, 5H, Ph), 7.46 (d, 1H, NH), 7.70 (d, 1H, NH).

Cbz-Ala-Ile-NH-NH-CH_2COOEt was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent; white solid, yield 55%. ^{1}H NMR

(DMSO-d_6): 0.72-0.82 (m, 6H, 2 x CH_3), 0.97 (m, 1H, CH), 1.14 (m, 6H, 2 x CH_3), 1.35 (m, 1H, CH), 1.60 (m, 1H, CH), 3.38 (s, 2H, NH*CH_2*CO), 4.03-4.19 (m, 4H, CO*CH_2*CH_3 and 2 x α-H), 4.99 (m, 2H, Cbz), 7.29-7.34 (m, 5H, Ph), 7.46 (d, 1H, NH), 7.66 (d, 1H, NH), 9.44 (s, 1H, NH).

Cbz-Ala-Phe-NH-NH-CH_2COOEt was purified by column chromatography on silica gel using 10% MeOH/CH_2Cl_2 as the eluent; white solid, yield 53%. ^{1}H NMR (DMSO-d_6): 1.09 (d, 3H, Ala-CH_3), 1.15-1.19 (t, 3H, NHCH$_2$COCH$_2$*CH_3*), 2.71 (s, 1H, NH) 2.76-2.92 (m, 2H, *CH_2*Ph), 3.33-3.39 (m, 2H, NH*CH_2*COCH$_2$CH$_3$) 3.98-4.08 (m, 3H, α-H and NHCH$_2$CO*CH_2*CH$_3$), 4.21 (s, 1H, NH), 4.43 (q, 1H, α-H), 4.99 (m, 2H, Cbz), 7.15-7.41 (m, 10H, 2 x Ph), 7.93 (d, 1H, NH), 9.13 (s, 1H, NH).

General Procedure for the Synthesis of Peptidyl-NH-NH-CH_2CONH$_2$. The peptidyl-NH-NHCH$_2$COOEt (1 eq) was dissolved in a 7 N solution (100 eq) of NH_3 in methanol and a small amount of DMF, and allowed to stir on an ice bath. To this solution was added catalytic NaCN (0.1 eq). The flask was closed with a rubber septum and allowed to stir at 4 °C for five days. The solvent was evaporated and the product was purified by column chromatography on a silica gel using the proper eluent gave the products with yields of 34-86%.

Boc-NH-NH-CH_2CONH$_2$ was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent; white solid, yield 80%. ^{1}H NMR (DMSO-d_6): 1.36 (s, 9H, Boc), 3.16 (s, 2H, N*CH_2*CO), 4.96 (d, 1H, NH), 7.16 (s, 1H, NH), 7.38 (s, 1H, NH), 8.24 (s, 1H, NH). MS (ESI) *m/z* 189.9 [(M + 1)$^+$].

Pip-Ala-NH-NH-CH_2CONH$_2$ was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent; white solid, yield 86%. ^{1}H NMR (DMSO-

d_6): 1.15 (d, 3H, Ala-CH_3), 1.38-1.41 (m, 4H, piperidine), 1.47-1.50 (m, 2H, piperidine), 3.17 (d, 2H, N*CH_2*CO), 3.23-3.26 (m, 4H, piperidine), 4.04 (q, 1H, α-H), 5.13 (d, 1H, NH), 5.36 (d, 1H, NH), 7.10 (s, 1H, NH), 7.46 (s, 1H, NH), 9.18 (d, 1H, NH). MS (ESI) *m/z* 272.0 [$(M + 1)^+$].

Mu-Ala-NH-NH-CH_2CONH_2 was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent; white solid, yield 53%. ^{1}H NMR (DMSO-d_6): 1.16 (d, 3H, Ala-CH_3), 3.18 (d, 2H, N*CH_2*CO), 3.25-3.26 (m, 4H, morpholine), 3.50-3.52 (m, 4H, morpholine), 4.02-4.09 (m, 1H, α-H), 5.14 (d, 1H, NH), 6.52 (d, 1H, NH), 7.12 (s, 1H, NH), 7.46 (s, 1H, NH), 9.23 (d, 1H, NH). MS (ESI) *m/z* 274.0 [$(M + 1)^+$].

Cbz-Piz-Ala-NH-NH-CH_2CONH_2 was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent; white solid, yield 62%. ^{1}H NMR (DMSO-d_6): 1.16 (d, 3H, Ala-CH_3) 3.18 (d, 2H, N*CH_2*CO), 3.31-3.33 (m, 8H, piperazine), 4.05 (m, 1H, α-H), 5.07 (s, 2H, Cbz), 5.15 (d, 1H, NH), 6.58 (d, 1H, NH), 7.11 (s, 1H, NH), 7.30-7.38 (m, 5H, Ph), 7.45 (s, 1H, NH), 7.85 (d, 1H, NH), 9.23 (d, 1H, NH).

Pip-Ala-Ala-NH-NH-CH_2CONH_2 was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent; white solid, yield 58%. ^{1}H NMR (DMSO-d_6): 1.15-1.18 (m, 6H, 2 x Ala-CH_3), 1.40 (s, 4H, piperidine), 1.48-1.51 (m, 2H, piperidine), 3.17 (d, 2H, N*CH_2*CO), 3.21-3.27 (m, 4H, piperidine), 4.06 (q, 1H, α-H), 4.13 (q, 1H, α-H), 5.19 (d, 1H, NH), 6.40 (d, 1H, NH), 7.11 (s, 1H, NH), 7.42 (s, 1H, NH), 7.79 (d, 1H, NH), 9.29 (d, 1H, NH). MS (ESI) *m/z* 343.1 [$(M + 1)^+$].

Mu-Ala-Ala-NH-NH-CH_2CONH_2 was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent; white solid, yield 48%. ^{1}H NMR

(DMSO-d_6): 1.16-1.19 (m, 6H, 2 x Ala-CH_3), 3.17 (d, 2H, N*CH_2*CO), 3.26-3.27 (m, 4H, morpholine), 3.50-3.53 (m, 4H, morpholine), 4.07 (q, 1H, α-H), 4.14 (q, 1H, α-H), 5.19 (d, 1H, NH), 6.54 (d, 1H, NH), 7.12 (s, 1H, NH), 7.42 (s, 1H, NH), 7.86 (d, 1H, NH), 9.27 (d, 1H, NH).

Boc-Piz-Ala-Ala-NH-NH-CH_2CONH_2 was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent; white solid, yield 67%. ^{1}H NMR (DMSO-d_6): 1.16-1.19 (m, 6H, 2 x Ala-CH_3), 1.39 (s, 9H, Boc), 3.17 (d, 2H, N*CH_2*CO), 3.27-3.33 (m, 8H, piperazine), 4.07 (q, 1H, α-H), 4.14 (q, 1H, α-H), 5.19 (d, 1H, NH), 6.57 (d, 1H, NH), 7.11 (s, 1H, NH), 7.41 (s, 1H, NH), 7.85 (d, 1H, NH), 9.26 (d, 1H, NH).

Cbz-Piz-Ala-Ala-NH-NH-CH_2CONH_2 was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent; white solid, yield 34%. ^{1}H NMR (DMSO-d_6): 1.15-1.19 (m, 6H, 2 x Ala-CH_3), 3.17 (d, 2H, N*CH_2*CO), 3.27-3.32-3.35 (m, 8H, piperazine), 4.07 (q, 1H, α-H), 4.14 (q, 1H, α-H), 5.07 (s, 2H, Cbz), 5.20 (d, 1H, NH), 6.60 (d, 1H, NH), 7.12 (s, 1H, NH), 7.23-7.35 (m, 5H, Ph), 7.41 (s, 1H, NH), 7.87 (d, 1H, NH), 9.27 (d, 1H, NH).

Cbz-Ala-Val-NH-NH-CH_2CONH_2 was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent; white solid, yield 41%. ^{1}H NMR (DMSO-d_6): 0.78-0.81 (m, 6H, 2 x Val-CH_3), 1.16 (d, 3H, Ala-CH_3), 1.85 (m, 1H, CH) 3.40 (d, 2H, NH*CH_2*CO), 4.09-4.13 (m, 2H, 2 x α-H), 4.99 (m, 2H, Cbz), 5.27 (d, 1H, NH) 7.13 (s, 1H, NH) 7.28-7.32 (m, 5H, Ph), 7.45 (s, 1H, NH), 7.73 (d, 1H, NH), 9.46 (d, 1H, NH).

Cbz-Ala-Ile-NH-NH-CH_2CONH_2 was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent; white solid, yield 25%. ^{1}H NMR (DMSO-d_6): 0.76-0.79 (m, 6H, 2 x Ile-CH_3), 1.02 (m, 1H, CH), 1.14 (d, 3H, Ala-CH_3), 1.40 (m, 1H, CH), 1.63 (m, 1H, CH), 3.18 (d, 2H, NH*CH_2*CO), 4.00-4.10 (m, 2H, 2 x α-H), 4.99 (m, 2H, Cbz), 5.29 (d, 1H, NH), 7.15-7.46 (m, 7H, Ph and 2 x NH), 7.77 (d, 1H, NH), 9.47 (d, 1H, NH).

Cbz-Ala-Phe-NH-NH-CH_2CONH_2 was purified by column chromatography on silica gel using 15% MeOH/CH_2Cl_2 as the eluent; white solid, yield 64%. ^{1}H NMR (DMSO-d_6): 1.10 (d, 3H, Ala-CH_3), 2.80-2.89 (m, 2H, *CH_2*Ph), 3.13 (d, 2H, NH*CH_2*CO) 4.00 (q, 1H, α-H), 4.38 (q, 1H, α-H), 4.99 (m, 2H, Cbz), 5.21 (d, 1H, NH), 7.12-7.40 (m, 12H, 2 x Ph and 2 x NH), 7.93 (s, 1H, NH), 7.97 (s, 1H, NH).

General Procedure for the Synthesis of Fumaric Acid Monoamides by the Mixed Anhydride Coupling Method. Coupling of the amine precursors to monoethyl fumarate was accomplished using the mixed anhydride coupling method. To a solution of the monoethyl fumarate (1 eq) in CH_2Cl_2 at -20 °C was added N-methylmorpholine (NMM, 1 eq) followed by isobutyl chloroformate (iBCF, 1 eq). After the reaction mixture was allowed to stir for 30 min, the amine (1 eq) was added to the mixture. Hydrochloride salts of the amine were pretreated with NMM (1 eq) at -20 °C in CH_2Cl_2 prior to addition. After 30 min the reaction was continued to stir overnight at room temperature. The methylene chloride was evaporated and the residue was redissolved in ethyl acetate and washed with 2% citric acid, saturated $NaHCO_3$, saturated NaCl, dried over $MgSO_4$, and concentrated. The product was purified by column chromatography as needed.

***trans*-3-Benzyloxycarbonylpropenoic Acid or Monobenzyl Fumarate (2b, HOOCCH=CHCOOBzl).** Equimolar amounts of fumaric acid and benzyl alcohol were dissolved in anhydrous DMF. NMM (1 eq) was added at 0 ºC followed by EDC after 15 minutes. The reaction was stirred over night at room temperature. DMF was evaporated and the crude residue was redissolved in EtOAc. The product was extracted with saturated aqueous $NaHCO_3$. The aqueous layer was then acidified with 1 N HCl to pH 2. The product was extracted with EtOAc, and the organic layer was washed with water and dried with $MgSO_4$. The solvent was evaporated and the crude residue was subjected to column chromatography to give a white powder (51% yield). ^{1}H-NMR (DMSO-d_6): 5.21 (s, 2H, CH=CH-COO*CH*$_2$Ph), 6.73 (s, 2H, *CH*=*CH*-COOCH$_2$Ph), 7.29-7.43 (m, 5H, Ph). MS (ESI) *m/z* 207 [$(M + 1)^+$].

***trans*-3-Cyclopropylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH=CHCONH-cyclopropyl)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and cyclopropylamine. Purification by column chromatography using 2:1 Hexanes:EtOAc as the eluent gave a white powder (84% yield). ^{1}H-NMR ($CDCl_3$): 0.56-0.59 (m, 2H, CH_2), 0.80-0.82 (m, 2H, CH_2), 1.28 (t, 3H, CH_3), 2.81-2.84 (m, 1H, CH), 4.20 (q, 2H, *CH*$_2$$CH_3$) 6.77-6.81 (d, 2H, *CH*=CHCON and NH), 6.90-6.94 (d, 1H, CH=*CH*CON).

***trans*-3-Cyclopropylcarbamoylpropenoic Acid (2c, HOOCCH=CHCONH-cyclopropyl).** EtOOCCH=CHCONH-cyclopropyl was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 eq) under standard deblocking conditions to give a white solid (91% yield). ^{1}H-NMR (DMSO, d_6): 0.41-0.45 (m, 2H, CH_2), 0.64-0.69 (m, 2H, CH_2), 2.69-

2.76 (m, 1H, CH), 6.45-6.49 (d, 1H, *CH*=CHCON), 6.78-6.62 (d, 1H, CH=*CH*CON), 8.53 (d, 1H, NH).

***trans*-3-Cyclohexylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH=CHCONH-cyclohexyl)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and cyclohexylamine. Purification by column chromatography using 2:1 EtOAc:hexane as the eluent gave a white powder (63% yield).

***trans*-3-Cyclohexylcarbamoylpropenoic Acid (2d, HOOCCH=CHCONH-cyclohexyl).** EtOOCCH=CHCONH-cyclohexyl was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 eq) under standard deblocking conditions to give a white solid (81% yield). ^{1}H-NMR (DMSO-d_6): 0.82-0.90 (m, 2H, CH_2), 1.07-1.39 (m, 4H, 2 x CH_2), 1.59-1.65 (m, 5H, 2 x CH_2 and CH), 2.98 (t, 2H, NH*CH*$_2$), 6.45-6.49 (d, 1H, *J* = 15.6, *CH*=CHCON), 6.91-6.95 (d, 1H, *J* = 15.6, CH=*CH*CON), 8.43 (t, 1H, NH).

***trans*-3-(2-Furyl)carbamoylpropenoic Acid Ethyl Ester (EtOOCCH=CHCONHCH$_2$-2-furyl)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and furylamine. Purification by column chromatography using 5% MeOH/CH_2Cl_2 as the eluent gave yellow oil. (84% yield). ^{1}H-NMR ($CDCl_3$): 1.26 (t, 3H, *CH*$_3$CH$_2$), 4.14-4.19 (q, 2H, CH$_3$*CH*$_2$), 4.50 (d, 2H, NH*CH*$_2$), 6.22-6.23 (d, 1H, furyl), 6.28-6.29 (d, 1H, furyl), 6.79-6.83 (d, 1H, *CH*=CHCON) 6.92 (t, 1H, NH) 6.98-7.02 (d, 1H, CH=*CH*CON), 7.32 (s, 1H, furyl).

***trans*-3-(2-Furyl)carbamoylpropenoic Acid (2e, HOOCCH=CHCONHCH$_2$-2-furyl).** EtOOCCH=CHCONHCH$_2$-2-furyl was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 eq) under standard deblocking conditions to give a white solid (79% yield). ^{1}H-NMR (DMSO-d_6): 4.38 (d, 2H, NH*CH*$_2$), 6.29-6.30 (d, 1H, furyl), 6.40-6.41 (d, 1H,

furyl), 6.53-6.57 (d, 1H, *CH*=CHCON), 6.93-6.97 (d, 1H, CH=*CH*CON), 7.60 (s, 1H, furyl), 8.96 (t, 1H, NH).

***trans*-3-Benzylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH=CHCONHBzl)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and benzylamine. Purification by column chromatography using 5% MeOH/CH_2Cl_2 gave a white powder (81% yield). ^{1}H-NMR (DMSO-d_6): 1.20-1.24 (t, 3H, CH_2CH_3), 4.14-4.19 (q, 2H, CH_2CH_3), 4.37 (d, 2H, NCH_2Ph), 6.57-6.61 (d, 1H, *CH*=CHCON), 7.03-7.07 (d, 1H, CH=*CH*CON), 7.22-7.34 (m, 5H, Ph), 9.04 (t, 1H, NH).

***trans*-3-Benzylcarbamoylpropenoic Acid (2f, HOOCCH=CHCONHBzl).** EtOOCCH=CHCONHBzl was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 eq) under standard deblocking conditions to give a white solid (73% yield). ^{1}H-NMR (DMSO-d_6): 4.36 (d, 2H, NCH_2Ph), 6.52-6.56 (d, 1H, *CH*=CHCON), 6.95-6.99 (d, 1H, CH=*CH*CON), 7.23-7.33 (m, 5H, Ph), 8.98 (t, 1H, NH). MS (ESI) *m/z* 206 [$(M + 1)^+$].

***trans*-3-Phenylethylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH=CHCONHCH$_2$CH$_2$Ph)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and phenylethylamine to give a clear colorless syrup (78% yield).

***trans*-3-Phenylethylcarbamoylpropenoic Acid (2g, HOOCCH=CHCONHCH$_2$CH$_2$Ph).** EtOOCCH=CHCONHCH$_2$CH$_2$Ph was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 eq) under standard deblocking conditions to give a clear, colorless syrup (81% yield). ^{1}H-NMR (DMSO-d_6): 3.54 (t, 2H, N-CH_2-CH_2-Ph),

3.61 (t, 2H, N-*CH*$_2$-CH$_2$-Ph) 6.43-6.47 (d, 1H, *J* = 15.2 Hz, *CH*=CHCON), 6.98-7.02 (d, 1H, *J* = 15.6 Hz, CH=*CH*CON), 7.14-7.31 (m, 5H, Ph).

***trans*-3-Phenylpropylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH=CHCONHCH$_2$CH$_2$CH$_2$Ph)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and 3-phenyl-1-propylamine. Purification by column chromatography using 1:1 EtOAc:hexane gave a white powder (80% yield).

***trans*-3-Phenylpropylcarbamoylpropenoic Acid (2h, HOOCCH=CHCONHCH$_2$CH$_2$CH$_2$Ph).** EtOOCCH=CHCONHCH$_2$CH$_2$CH$_2$Ph was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 eq) under standard deblocking conditions to give a white powder (81% yield). ^{1}H-NMR (DMSO-d$_6$): 1.68-1.76 (m, 2H, NH-CH$_2$-*CH*$_2$-CH$_2$-Ph), 2.49-2.59 (t, 2H, NH-CH$_2$-CH$_2$-*CH*$_2$-Ph), 3.12-3.17 (q, 2H, NH-*CH*$_2$-CH$_2$-CH$_2$-Ph) 6.47-6.51 (d, 1H, *J* = 15.6 Hz, *CH*=CHCON), 6.90-6.94 (d, 1H, *J* = 15.6 Hz, CH=*CH*CON), 7.15-7.28 (m, 5H, Ph), 8.51 (t, 1H, NH).

***trans*-3-(3,4-Dimethoxybenzylcarbamoyl)propenoic Acid Ethyl Ester (EtOOCCH =CH-CONHCH$_2$Ph-3,4-OCH$_3$)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and 3,4-dimethoxyphenylmethanamine. Purification by column chromatography using 5% MeOH/CH$_2$Cl$_2$ gave a white powder (69% yield). ^{1}H-NMR (DMSO-d$_6$): 1.22 (t, 3H, CH$_3$), 3.70-3.72 (d, 6H, 2 x OCH$_3$), 4.16 (q, 2H, *CH*$_2$CH$_3$), 4.29 (d, 2H, NH*CH*$_2$), 6.57-6.61 (d, 1H, *CH*=CHCON), 6.76-6.79 (d, 1H, Ph), 6.87-6.89 (d, 2H, Ph), 7.02-7.06 (d, 1H, CH=*CH*CON), 8.92 (t, 1H, NH).

***trans*-3-(3,4-Dimethoxybenzylcarbamoyl)propenoic Acid (2i, HOOCCH =CH-CONHCH$_2$Ph-3,4-OCH$_3$).** EtOOCCH =CH-CONHCH$_2$Ph-3,4-OCH$_3$ was

hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 eq) under standard deblocking conditions to give a white powder (72% yield).

***trans*-3-(3,4-Dihydro-2H-quinolin-1-ylcarbonyl)propenoic Acid Ethyl Ester (EtOOCCH=CHCO-tetrahydroquinoline)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and 1,2,3,4-tetrahydroquinoline to give a brown syrup (83% yield). ^{1}H-NMR ($CDCl_3$): 1.29 (t, 3H, CH_3CH_2OC), 1.99-2.02 (m, 2H, N-CH_2-CH_2-CH_2), 2.73-2.76 (t, 1H, N-CH_2-CH_2-CH_2), 3.86-3.98 (t, 1H, N-CH_2-CH_2-CH_2), 4.24-4.30 (q, 2H, CH_3CH_2OOC), 6.78-6.82 (dd, 1H, J = 14.8 Hz, *CH*=CHCON), 7.18-7.22 (m, 4H, quinoline), 7.44-7.48 (d, 1H, J = 14.8 Hz, CH=*CH*CON).

***trans*-3-(3,4-Dihydro-2H-quinolin-1-ylcarbonyl)propenoic Acid (2j, HOOCCH=CHCO-tetrahydroquinoline).** EtOOCCH=CHCO-tetrahydroquinoline was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 eq) under standard deblocking conditions to give a clear syrup, which was recrystallized using cold EtOAc to give a yellow powder. (78% yield). ^{1}H-NMR (DMSO-d_6): 1.84-1.91 (m, 2H, N-CH_2-CH_2-CH_2), 2.68-2.71 (t, 1H, N-CH_2-CH_2-CH_2), 3.71-3.75 (t, 1H, N-CH_2-CH_2-CH_2), 6.56-6.60 (d, 1H, J = 15.6 Hz, *CH*=CHCON), 7.06-7.24 (m, 5H, quinoline and CH=*CH*CON).

***trans*-3-(3,4-Dihydro-2H-quinolin-1-ylcarbonyl)propenoic Acid Ethyl Ester (EtOOCCH=CHCO-tetrahydroisoquinoline)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and 1,2,3,4-tetrahydroisoquinoline to give a brown syrup (83% yield). ^{1}H-NMR ($CDCl_3$): 1.29 (t, 3H, CH_3CH_2OC), 1.99-2.02 (m, 2H, N-CH_2-CH_2-CH_2), 2.73-2.76 (t, 1H, N-CH_2-CH_2-CH_2), 3.86-3.98 (t, 1H, N-CH_2-CH_2-CH_2), 4.24-4.30 (q, 2H, CH_3CH_2OOC), 6.78-6.82

(dd, 1H, *J* = 14.8 Hz, *CH*=CHCON), 7.18-7.22 (m, 4H, quinoline), 7.44-7.48 (d, 1H, *J* = 14.8 Hz, CH=*CH*CON).

***trans*-3-(3,4-Dihydro-2H-quinolin-1-ylcarbonyl)propenoic Acid (2k, HOOCCH=CHCO-tetrahydroisoquinoline).** EtOOCCH=CHCO-tetrahydroquinoline was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 eq) under standard deblocking conditions to give a clear syrup, which was recrystallized using cold EtOAc to give a yellow powder (68% yield). ^{1}H-NMR (DMSO-d_6): 1.2.78-2.87 (m, 2H, $CH_2NCH_2CH_2$), 3.71-3.78 (m, 2H, $CH_2NCH_2CH_2$), 4.66-4.76 (d, 2H, $CH_2NCH_2CH_2CH_2$), 6.50-6.54 (d, 1H, *CH*=CHCON), 7.16-7.21 (m, 4H, quinoline), 7.43-7.49 (d, 1H, CH=*CH*CON).

***trans*-3-Dibenzylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH=CHCON(Bzl)$_2$)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and dibenzylamine to give a clear, pink syrup (87% yield).

***trans*-3-Dibenzylcarbamoylpropenoic Acid (2l, HOOCCH=CHCON(Bzl)$_2$).** EtOOCCH=CHCON(Bzl)$_2$ was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 eq) under standard deblocking conditions to give a white powder (91% yield). ^{1}H-NMR (DMSO-d_6): 4.57 (s, 2H, NCH_2Ph), 4.65 (s, 2H, NCH_2Ph), 6.61-6.65 (d, 1H, *CH*=CHCON), 7.15-7.17 (d, 1H, CH=*CH*CON), 7.25-7.50 (m, 10H, 2 x Ph).

***trans*-3-(1-Naphthylmethylcarbamoyl)propenoic Acid Ethyl Ester (EtOOCCH=CH-CONHCH$_2$-1-Naphth)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and 1-naphthylmethylamine. Purification by column chromatography using 2:1 Hexanes:EtOAc to give white powder (86% yield). ^{1}H-NMR ($CDCl_3$): 1.20 (t, 3H, CH_3), 3.99 (q, 2H, CH_2), 4.93 (d, 2H, CH_2-naphth), 6.84

(d, 1H, *CH*=CHCON), 6.90 (d. 1H, CH=*CH*CON), 7.38-7.44 (m, 2H, naphthyl), 7.47-7.54 (m, 2H, naphthyl), 7.79 (d, 1H, naphthyl), 7.84 (d, 1H, naphthyl), 7.95 (d, 1H, naphthyl).

***trans*-3-(1-Naphthylmethylcarbamoyl)propenoic Acid (2m, HOOCCH=CH-CONHCH$_2$-1-Naphth).** EtOOCCH=CHCONHCH$_2$-1-naphth was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 eq) under standard deblocking conditions to give a white solid (76% yield). ^{1}H-NMR (DMSO-d$_6$): 4.82 (d, 2H, *CH$_2$*-naphth), 6.56-6.60 (d, 1H, *J* = 15.6 Hz, C*H*=CHCON), 6.91-6.95 (d. 1H, , *J* = 15.6 Hz, CH=*CH*CON), 7.40-7.57 (m, 4H, naphthyl), 7.84-7.86 (m, 1H, naphthyl), 7.94 (d, 1H, naphthyl), 8.03 (d, 1H, naphthyl), 9.00 (t, 1H, NH).

***trans*-3-(Methyl-1-naphthylmethylcarbamoyl)propenoic Acid Ethyl Ester (EtOOCCH=CHCON(CH$_3$)CH$_2$-1-Naphth)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and N-methyl-1-naphthylmethylamine hydrochloride. Purification by column chromatography using 1:1 EtOAc:hexane gave a white powder (62% yield). ^{1}H-NMR (CDCl$_3$): 1.29 (t, 3H, CH$_3$), 3.01 (s, 3H, CH$_3$), 5.11 (s, 2H, CH$_2$), 6.87-6.92 (d, 1H, *CH*=CHCON), 7.40-7.52 (m, 5H, CH=*CH*CON and naphthyl), 7.78-7.89 (m, 3H, naphthyl).

***trans*-3-(Methyl-1-naphthylmethylcarbamoyl)propenoic Acid (2n, HOOCCH=CHCON(CH$_3$)CH$_2$-1-Naphth).** EtOOCCH=CHCON(CH$_3$)CH$_2$-1-Napth was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 eq) under standard deblocking conditions to give a white solid (13% yield). ^{1}H-NMR (DMSO-d$_6$): 3.01 (s, 3H, CH$_3$), 5.01 (s, 2H, CH$_2$), 6.61-6.65 (d, 1H, *J* = 15.2 Hz, *CH*=CHCON), 7.17-7.21 (d, 1H, CH=*CH*CON), 7.37-7.60 (m, 4H, naphthyl), 7.85-8.01 (m, 3H, naphthyl).

***trans*-3-(Benzyl-1-naphthylmethylcarbamoyl)propenoic Acid Ethyl Ester (EtOOCCH=CHCON(Bzl)-CH_2-1-Naphth)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and benzyl-1 naphthylmethylamine. Purification by column chromatography using 1:1 EtOAc:hexane gave a yellow oil (89% yield). ^{1}H-NMR ($CDCl_3$): 1.31 (t, 3H, *CH_3*CH_2), 4.23 (q, 2H, CH_3*CH_2*), 4.55-4.69 (d, 2H, N-CH_2), 4.71-4.81 (d, 2H, N-CH_2), 6.91-6.95 (d, 1H, *J* = 14.4 Hz, *CH*=CHCON), 7.16-7.18 (d, 1H, CH=*CH*CON), 7.23-7.57 (m, 10H, naphthyl and Ph), 7.85 (m, 2H, naphthyl).

***trans*-3-(Benzyl-1-naphthylmethylcarbamoyl)propenoic Acid (2o, HOOCCH=CHCON(Bzl)-CH_2-1-Naphth).** EtOOCCH=CHCON(Bzl)-1-CH_2-Napth was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 eq) under standard deblocking conditions to give a white solid after recrystallization from cold EtOAc (77% yield). ^{1}H-NMR (DMSO-d_6): 4.82 (d, 2H, N-CH_2), 5.01 (d, 2H, N-CH_2), 6.61-6.65 (d, 1H, *J* = 15.2 Hz, *CH*=CHCON), 7.17-7.21 (d, 1H, CH=*CH*CON), 7.37-7.60 (m, 9H, naphthyl and Ph), 7.85-8.01 (m, 3H, naphthyl).

***trans*-3-(Di-1-naphthylmethylcarbamoyl)propenoic Acid Ethyl Ester (EtOOCCH=CHCON(CH_2-1-Naphth)$_2$)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and *N,N*-di(1-naphthylmethyl)amine. Purification by column chromatography using 2:1 Hexanes:EtOAC gave white solid (40% yield). ^{1}H-NMR (DMSO-d_6): 1.10 (t, 3H, CH_3), 4.04 (q, 2H, CH_2), 5.22 (s, 4H, N-1-*CH_2*-Napth), 6.73-6.77 (d, 1H, *CH*=CHCON), 7.18-7.19 (d, 1H, CH=*CH*CON), 7.17-8.11 (m, 14H, N(1-CH_2-*Napth*)$_2$).

***trans*-3-(Di-1-naphthylmethylcarbamoyl)propenoic Acid (2p, HOOCCH=CHCON(CH_2-1-Naphth)$_2$).** EtOOCCH=CHCON(CH_2-1-Napth)$_2$) was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 eq) under standard deblocking conditions to give a white solid (69% yield). ^{1}H-NMR (DMSO-d_6): 5.21 (s, 4H, N-1-*CH_2*-Napth), 6.61-6.62 (d, 1H, *CH*=CHCON), 6.69-6.72 (d, 1H, CH=*CH*CON), 7.19-8.10 (m, 14H, N(1-CH_2-*Napth*)$_2$).

(*2S,3S*)-Oxirane-2,3-dicarboxylic Acid Monoethyl Esters (4q, Monoethyl Epoxysuccinates, HOOC-EP-COOEt). The diethyl trans epoxysuccinates were synthesized stereoselectively by a procedure adapted from one described previously by Mori and Iwasawa.[67] One ethyl ester was hydrolyzed using 1M KOH (1 eq) in EtOH at 0 °C. ^{1}H NMR (DMSO-d_6): 1.19 (t, 3H, OCH$_2$*CH_3*), 3.55 (d, 1H, epoxy CH), 3.64 (d, 1H, epoxy CH), 4.15 (q, 2H, O*CH_2*CH$_3$).

***cis*-Oxirane-2,3-dicarboxylic Acid Monoethyl Ester (Monoethyl Epoxysuccinate, HOOC-EP-COOEt).** The diethyl ester cis epoxysuccinate was synthesized using the procedure described by Meth-Cohn.[68] One ethyl ester was selectively hydrolyzed as described by Rich[69] and Schaschke.[70] ^{1}H NMR ($CDCl_3$): 1.27-1.36 (m, 3H, OCH$_2$*CH_3*), 3.75-3.81 (dd, 2H, epoxy CH), 4.25-4.37 (m, 2H, O*CH_2*CH$_3$).

General Procedure for Coupling of the Mono Ethyl Ester Epoxysuccinates to Amines. The method used was the mixed anhydride coupling method. The mono ethyl ester epoxysuccinate (1 eq) was dissolved in CH_2Cl_2 and cooled to -20 °C. To the reaction mixture was added NMM (3 eq) and then iBCF (3 eq). The reaction mixture was stirred at -20 °C for 15-20 minutes and then the amine (3 eq) was added. The reaction mixture was then stirred at -20 °C for one hour and then at room temperature

overnight. The solvent was removed, and the crude product was dissolved in EtOAc. The organic layer was then washed with of 2% citric acid, saturated $NaHCO_3$ and brine. The product was purified with column chromatography as needed. Hydrolysis of the ethyl ester with 1 M NaOH (1.5 eq) in EtOH gave the desired amides.

(*2S,3S*)-Oxirane-2,3-dicarboxylic Acid Dibenzyl Amide (4r, HOOC-EP-CON(Bzl)$_2$) was obtained by mixed anhydride coupling of the mono ethyl ester epoxysuccinate and dibenzylamine to give a clear oil and then hydrolyzed in basic conditions to the corresponding acid. ^{1}H NMR (DMSO-d_6): 3.51 (d, 1H, epoxy CH), 4.03 (d, 1H, epoxy CH), 4.50 (dd, 2H, N*CH*$_2$Ph), 4.70 (dd, 2H, NCH$_2$Ph), 7.18-7.36 (m, 10H, 2 x Ph).

(*2S,3S*)-Oxirane-2,3-dicarboxylic Acid 1-Naphthylmethyl Amide (4s, HOOC-EP-CONH-1-CH$_2$-Napth) was obtained by mixed anhydride coupling of the mono ethyl ester epoxysuccinate and 1-naphthylmethylamine to give a yellow powder and then hydrolyzed in basic conditions to the corresponding acid. ^{1}H NMR (Acetone-d_6): 3.59 (d, 1H, epoxy CH), 3.82 (d, 1H, epoxy CH), 5.21-5.36 (m, 4H, N(1-*CH*$_2$-Napth)$_2$), 7.37-7.56 (m, 8H, N(1-CH$_2$-*Napth*)$_2$), 7.84-7.96 (m, 5H, N(1-CH$_2$-*Napth*)$_2$), 8.15-8.18 (m, 1H, N(1-CH$_2$-*Napth*)$_2$).

(*cis*)-Oxirane-2,3-dicarboxylic Acid Dibenzyl Amide (6t, HOOC-EP-CON(Bzl)$_2$) was obtained by mixed anhydride coupling of the mono ethyl ester epoxysuccinate and dibenzylamine to give a clear oil and then hydrolyzed in basic conditions to the corresponding acid. ^{1}H NMR (DMSO-d_6): 3.79 (d, 1H, epoxy CH), 4.13 (d, 1H, epoxy CH), 4.23-4.63 (dd, 4H, N*CH*$_2$Ph), 7.16 (d, 2H, Ph), 7.24-7.37 (m, 8H, Ph).

General Procedure for the Synthesis of Aza-Peptide Michael Acceptors and Aza-Peptide Epoxides by the HOBt and EDC Coupling Method. To a stirred solution of the fumaric acid or epoxide precursor (1.5 eq) in DMF at -10 °C was added HOBt (1.5 eq), the peptidyl hydrazide precursor (1 eq) and EDC (1.5 eq) was added. The mixture was allowed to react for 16 h at room temperature. The DMF was evaporated, and the residue was redissolved in EtOAc. The organic layer was washed with 2% citric acid, saturated $NaHCO_3$, saturated NaCl, dried over $MgSO_4$, and concentrated. Column chromatography on silica gel afforded the aza-peptidyl fumarate and aza-peptidyl epoxide derivatives.

N^2-*tert*-butoxycarbonyl-N^1-carbamoylmethyl-N^1-*trans*-(3-ethoxycarbonylpropenoyl)hydrazine (23a, Boc-AAsn-CH=CH-COOEt). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (47% yield). ^{1}H-NMR (DMSO-d_6): 1.19 (t, 3H, CH_3), 1.39 (s, 9H, Boc), 3.59 (d, 1H, CH), 4.17 (q, 2H, CH_2), 4.43 (d, 1H, CH), 6.59 (d, 1H, *CH*=CHCON), 7.19-7.25 (m, 3H, CH=*CH*CON and NH), 7.43 (s, 1H, NH), 9.93 (s, 1H, NH). MS (FAB) *m/z* 316.1 [$(M + 1)^+$]. Anal. Calcd. for $C_{13}H_{21}N_3O_6 \cdot 0.15CH_2Cl_2 \cdot 0.2H_2O$: C, 47.62; H, 6.59; N, 12.67. Found: C, 47.61; H, 6.32; N, 12.48.

N^2-*tert*-butoxycarbonyl-N^1-carbamoylmethyl-N^1-*trans*-(3-cyclohexylcarbamoylpropenoyl)hydrazine (23d, Boc-AAsn-CH=CH-$NHCH_2$-cyclohexyl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent.

Recrystallization with EtOAc/hexane gave a white powder (24% yield). ^{1}H-NMR (DMSO-d_6): 0.84-0.89 (m, 2H, cyclohexyl), 1.12-1.19 (m, 4H, cyclohexyl), 1.39 (s, 9H, Boc), 1.42-1.64 (m, 5H, cyclohexyl), 2.98 (t, 2H, CH_2), 3.57 (d, 1H, CH), 4.38 (d, 1H, CH), 6.89 (d, 1H, *CH*=CHCON), 7.08 (d, 1H, CH=*CH*CON), 7.17 (s, 1H, NH), 7.41 (s, 1H, NH), 8.43 (t, 1H, NH), 9.88 (s, 1H, NH). MS (FAB) *m/z* 383.2 [$(M + 1)^+$]. Anal. Calcd. for $C_{18}H_{30}N_4O_5 \cdot 0.15H_2O$: C, 56.13; H, 7.93; N, 14.55. Found: C, 56.14; H, 7.89; N, 14.46.

N^2-*tert*-butoxycarbonyl-N^1-carbamoylmethyl-N^1-*trans*-(3-benzylcarbamoylpropenoyl)hydrazine (23f, Boc-AAsn-CH=CH-CONHBzl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (44% yield). ^{1}H-NMR (DMSO-d_6): 0.84-0.89 (m, 2H, cyclohexyl), 1.12-1.19 (m, 4H, cyclohexyl), 1.39 (s, 9H, Boc), 3.59 (d, 1H, CH), 4.35-4.41 (m, 3H, CH and CH_2), 6.92-6.96 (d, 1H, *CH*=CHCON), 7.14-7.33 (m, 7H, Ph and CH=*CH*CON and NH), 7.41 (s, 1H, NH), 8.98 (t, 1H, NH), 9.89 (s, 1H, NH). MS (FAB) *m/z* 377.2 [$(M + 1)^+$]. Anal. Calcd. for $C_{18}H_{24}N_4O_5$: C, 57.44; H, 6.43; N, 14.88. Found: C, 57.50; H, 6.52; N, 14.73.

N^2-*tert*-butoxycarbonyl-N^1-carbamoylmethyl-N^1-*trans*-(3-phenylpropylcarbamoylpropenoyl)hydrazine (23h, Boc-AAsn-CH=CH-$CONHCH_2CH_2CH_2Ph$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (53% yield). ^{1}H-NMR (DMSO-d_6): 1.39 (s, 9H, Boc), 1.67-1.74 (m, 2H, $NHCH_2CH_2CH_2Ph$), 2.55-2.58 (t, 2H,

NHCH$_2$CH$_2$*CH$_2$*Ph), 3.11-3.16 (m, 2H, NH*CH$_2$*CH$_2$CH$_2$Ph), 3.59 (d, 1H, CH), 4.41 (d, 1H, NH), 6.87-6.91 (d, 1H, *CH*=CHCON), 7.08-7.28 (m, 7H, Ph and CH=*CH*CON and NH), 7.41 (s, 1H, NH), 8.51 (t, 1H, NH), 9.89 (s, 1H, NH). MS (FAB) *m/z* 405.3 [(M + 1)$^+$]. Anal. Calcd. for $C_{20}H_{28}N_4O_5 \cdot 1.2$Hexane: C, 60.18; H, 7.29; N, 13.24. Found: C, 60.09; H, 7.39; N, 12.73.

N^2-(N-Piperidinocarbonylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(3,4-dihydro-1H-isoquinolin-2-ylcarbonyl)propenoyl)hydrazine (24k, Pip-Ala-AAsn-CH=CHCO-tetrahydroisoquinoline). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (41% yield). ^{1}H-NMR (DMSO-d_6): 1.23 (d, 3H, CH_3), 1.37 (s, 4H, piperidine), 1.48 (2H, piperidine), 1.74-1.77 (m, 2H, N-CH$_2$-*CH$_2$*-CH$_2$), 2.79-2.87 (m, 2H, N-CH$_2$-CH$_2$-*CH$_2$*), 3.23 (m, 4H, piperidine), 3.60 (s, 2H, NCH$_2$CO), 3.72-3.75 (m, 2H, N-*CH$_2$*-CH$_2$), 4.12 (m, 1H, α-H), 6.55 (d, 1H, NH), 7.06-7.37 (m, 7H, isoquinoline and *CH*=CHCON and NH and CH=*CH*CON), 7.57 (s, 1H, NH), 10.53 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{24}H_{33}N_6O_5$: 485.2507. Observed *m/z* 485.2579. Anal. Calcd. for $C_{24}H_{32}N_6O_5 \cdot 1.3H_2O$: C, 56.75; H, 6.87; N, 16.54. Found: C, 56.80; H, 6.79; N, 16.73.

N^2-(N-Morpholinocarbonylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-cyclopropylcarbamoylpropenoyl)hydrazine (25c, Mu-Ala-AAsn-CH=CH-CONH-cyclopropyl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (67% yield). ^{1}H-NMR (DMSO-d_6): 0.40-0.43 (m, 2H, CH_2), 0.63-0.68 (m, 2H, CH_2), 1.24 (d, 3H, CH_3), 2.69-

2.73 (m, 1H, CH), 3.28-3.29 (m, 4H, morpholine), 3.33 (s, 2H, N*CH*$_2$CO), 3.51-3.53 (m, 4H, morpholine), 4.11-4.18 (m, 1H, α-H), 6.71-6.77 (m, 2H, NH and *CH*=CHCON), 7.03-7.07 (d, 1H, CH=*CH*CON), 7.17 (s, 1H, NH), 7.53(s, 1H, NH), 8.47 (d, 1H, NH), 10.58 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{17}H_{27}N_6O_6$: 411.1987. Observed *m/z* 411.1981. Anal. Calcd. for $C_{17}H_{26}N_6O_6 \cdot 1H_2O$: C, 47.66; H, 6.59; N, 19.62. Found: C, 47.60; H, 6.46; N, 19.48.

N^2-(N-Morpholinocarbonylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(2-furyl)carbamoylpropenoyl)hydrazine (25e, Mu-Ala-AAsn-CH=CH-CONHCH$_2$-2-furyl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a yellow powder (48% yield). ^{1}H-NMR (DMSO-d_6): 1.24 (d, 3H, CH_3), 3.27-3.28 (m, 4H, morpholine), 3.34 (s, 2H, N*CH*$_2$CO), 3.50-3.52 (m, 4H, morpholine), 4.11-4.18 (m, 1H, α-H), 4.34 (d, 2H, NH*CH*$_2$), 6.24-6.25 (d, 1H, furyl), 6.37-6.38 (d, 1H, furyl), 6.71 (d, 1H, NH), 6.84-6.88 (d, 1H, *CH*=CHCON), 7.09-7.13 (d, 1H, CH=*CH*CON), 7.18 (s, 1H, NH), 7.54-7.56 (d, 2H, NH and furyl), 8.86 (t, 1H, NH), 10.59 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{19}H_{27}N_6O_7$: 451.1936. Observed *m/z* 451.2037. Anal. Calcd. for $C_{19}H_{26}N_6O_7 \cdot 1.3H_2O$: C, 48.16; H, 6.08; N, 17.74 . Found: C, 48.16; H, 6.09; N, 17.54.

N^2-(N- Morpholinocarbonylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(3,4-dimethoxybenzyl)carbamoylpropenoyl)hydrazine (25i, Mu-Ala-AAsn-CH=CH-CONHCH$_2$Ph-3,4-OCH$_3$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (14% yield). ^{1}H-NMR

(DMSO-d_6): 1.24 (d, 3H, CH_3), 3.25-3.26 (m, 4H, morpholine), 3.32 (s, 2H, N*CH_2*CO), 3.49-3.51 (m, 4H, morpholine), 3.70-3.71 (d, 6H, 2 x OCH_3), 4.11-4.18 (m, 1H, α-H), 4.28 (d, 2H, NH*CH_2*), 6.70-6.72 (d, 1H, NH), 6.74-6.76 (d, 1H, *CH*=CHCON), 6.86-6.91 (m, 3H, Ph and CH=*CH*CON), 7.10-7.18 (m, 2H, Ph and NH), 7.53 (s, 1H, NH), 8.82 (t, 1H, NH), 10.59 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{23}H_{33}N_6O_8$: 521.2354. Observed *m/z* 521.2431. Anal. Calcd. for $C_{23}H_{32}N_6O_8 \cdot 0.1EtOAc \cdot 1H_2O$: C, 51.35; H, 6.41; N, 15.35. Found: C, 51.99; H, 6.45; N, 15.75.

N^2-(N-Morpholinocarbonylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-phenylpropylcarbamoylpropenoyl)hydrazine (25h, Mu-Ala-AAsn-CH=CH-$CONHCH_2CH_2CH_2Ph$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (41% yield). ^{1}H-NMR (DMSO-d_6): 1.24 (d, 3H, CH_3), 1.67-1.75 (m, 2H, $NHCH_2CH_2CH_2Ph$), 2.55-2.59 (t, 2H, $NHCH_2CH_2CH_2Ph$), 3.09-3.18 (m, 2H, $NHCH_2CH_2CH_2Ph$), 3.26-3.28 (m, 4H, morpholine), 3.33 (s, 2H, N*CH_2*CO), 3.50-3.52 (m, 4H, morpholine), 4.11-4.18 (m, 1H, α-H), 6.71 (d, 1H, NH), 6.84-6.88 (d, 1H, *CH*=CHCON), 7.09-7.14 (d, 1H, CH=*CH*CON), 7.15-7.28 (m, 6H, Ph and NH), 7.53 (s, 1H, NH), 8.45 (t, 1H, NH), 10.58 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{23}H_{33}N_6O_6$: 489.2462. Observed *m/z* 489.2486. Anal. Calcd. for $C_{23}H_{32}N_6O_6 \cdot 0.5H_2O$: C, 55.52; H, 6.68; N, 16.89. Found: C, 55.51; H, 6.65; N, 16.80.

N^2-(N-Morpholinocarbonylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(3,4-dihydro-1H-isoquinolin-2-ylcarbonyl)propenoyl)hydrazine (25k, Mu-Ala-AAsn-CH=CHCO-tetrahydroisoquinoline). This compound was obtained using the

HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (27% yield). ^{1}H-NMR (DMSO-d_6): 1.23 (d, 3H, Ala-CH_3), 2.78-2.86 (m, 2H, N-CH_2-*CH_2*-CH_2), 3.22-3.34 (m, 6H, morpholine and N*CH_2*CO), 3.47-3.58 (m, 6H, N-CH_2-CH_2-*CH_2* and morpholine), 3.71-3.73 (m, 2H, N-*CH_2*-CH_2), 4.13 (m, 1H, α-H), 6.69 (d, 1H, NH), 7.05-7.39 (m, 7H, *CH*=CHCON and CH=*CH*CON and isoquinoline and NH), 7.55 (s, 1H, NH), 10.58 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{23}H_{31}N_6O_6$: 485.2507. Observed *m/z* 485.2579. Anal. Calcd. for $C_{23}H_{30}N_6O_6 \cdot 1.4H_2O$: C, 53.98; H, 6.46; N, 16.42. Found: C, 54.03; H, 6.39; N, 16.03.

N^2-(4-(Benzyloxycarbonyl)piperazin-1-ylcarbonylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(3,4-dihydro-2H-quinolin-1-ylcarbonyl)propenoyl)hydrazine (26j, Cbz-Piz-Ala-AAsn-CH=CHCO-tetrahydroquinoline). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a yellow powder (29% yield). ^{1}H-NMR (DMSO-d_6): 1.27 (m, 3H, Ala-CH_3), 1.84-1.89 (m, 2H, N-CH_2-*CH_2*-CH_2), 2.67-2.71 (m, 2H, N-CH_2-CH_2-*CH_2*), 3.33 (m, 10H, piperazine and N*CH_2*CO), 3.70-3.76 (m, 2H, N-*CH_2*-CH_2), 4.13-4.20 (m, 1H, α-H), 5.07 (s, 2H, Cbz), 6.79 (d, 1H, NH), 7.05-7.37 (m, 12H, Ph, quinoline, NH, *CH*=CHCON and CH=*CH*CON), 7.53 (s, 1H, NH), 10.60 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{31}H_{38}N_7O_7$: 620.2827. Observed *m/z* 620.2891. Anal. Calcd. for $C_{31}H_{37}N_7O_7 \cdot 0.9H_2O$: C, 58.55; H, 6.15; N, 15.42. Found: C, 58.68; H, 6.14; N, 15.22.

N^2-(4-(Benzyloxycarbonyl)piperazin-1-ylcarbonylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(3,4-dihydro-1H-isoquinolin-2-ylcarbonyl)propenoyl)hydrazine (26k, Cbz-Piz-Ala-AAsn-CH=CHCO-tetrahydroisoquinoline). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (46% yield). ^{1}H-NMR (DMSO-d_6): 1.23 (d, 3H, CH_3), 2.75-2.87 (m, 2H, N-CH_2-CH_2-*CH_2*), 3.30-3.33 (m, 10H, piperazine and N*CH_2*CO), 3.69-3.77 (m, 2H, N-*CH_2*-CH_2), 4.10-4.15 (m, 1H, α-H), 4.64-4.74 (m, 2H, N*CH_2*C), 5.07 (s, 2H, Cbz), 6.76 (d, 1H, NH), 7.05-7.39 (m, 12H, Ph, isoquinoline, NH, *CH*=CHCON and CH=*CH*CON), 7.55 (s, 1H, NH), 10.57 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{31}H_{38}N_7O_7$: 620.2827. Observed *m/z* 620.2879. Anal. Calcd. for $C_{31}H_{37}N_7O_7 \cdot 1H_2O \cdot 0.5EtOAc$: C, 58.14; H, 6.36; N, 14.38. Found: C, 58.48; H, 6.33; N, 14.62.

N^2-(N-Piperidinocarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-ethoxycarbonylpropenoyl)hydrazine (27a, Pip-Ala-Ala-AAsn-CH=CH-COOEt). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (35% yield). ^{1}H-NMR (DMSO-d_6): 1.17-1.25 (m, 9H, 2 x Ala-CH_3 and $COCH_2$*CH_3*), 1.39 (s, 4H, piperidine), 1.49 (m, 2H, piperidine), 3.25 (s, 4H, piperidine), 3.32 (s, 2H, N*CH_2*CO), 4.09-4.19 (m, 3H, α-H and CO*CH_2*CH_3), 4.25 (m, 1H, α-H), 6.35 (d, 1H, NH), 6.59 (d, 1H, *CH*=CHCON), 7.18-7.20 (m, 2H, CH=*CH*CON and NH), 7.51 (s, 1H, NH), 8.04 (br s, 1H, NH), 10.75 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{20}H_{33}N_6O_7$: 469.2405. Observed *m/z* 469.2426. Anal. Calcd.

for $C_{20}H_{32}N_6O_7 \cdot 0.9H_2O$: C, 49.56; H, 7.03; N, 17.34. Found: C, 49.60; H, 7.04; N, 17.29.

N^2-(N-Piperidinocarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-phenylethylcarbamoylpropenoyl)hydrazine (27g, Pip-Ala-Ala-AAsn-CH=CH-CONHCH$_2$CH$_2$Ph). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (48% yield). ^{1}H-NMR (DMSO-d_6): 1.19-1.28 (m, 6H, 2 x CH_3), 1.39 (s, 4H, piperidine), 1.50 (m, 2H, piperidine), 2.75 (t, 2H, CH_2), 3.25-3.28 (m, 4H, piperidine), 3.34 (s, 2H, N*CH$_2$*CO), 3.35-3.40 (m, 2H, CH_2), 4.13 (m, 1H, α-H), 4.27 (m, 1H, α-H), 6.41 (d, 1H, NH), 6.83-6.87 (d, 1H, *CH*=CHCON), 7.03-7.07 (d, 1H, CH=*CH*CON), 7.18-7.31 (m, 6H, Ph and NH), 7.52 (s, 1H, NH), 8.07 (s, 1H, NH), 8.56 (t, 1H, NH), 10.69 (br s, 1H, NH). HRMS (FAB) Calcd. for $C_{26}H_{38}N_7O_6$: 544.2878. Observed *m/z* 544.3001. Anal. Calcd. for $C_{26}H_{37}N_7O_6 \cdot 0.1H_2O$: C, 57.26; H, 6.87; N, 17.98. Found: C, 57.31; H, 6.83; N, 17.94.

N^2-(N-Piperidinocarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-dibenzylcarbamoylpropenoyl)hydrazine (27l, Pip-Ala-Ala-AAsn-CH=CH-CON(Bzl)$_2$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (52% yield). ^{1}H-NMR (DMSO-d_6): 1.18-1.27 (m, 6H, 2 x Ala-CH_3), 1.37 (s, 4H, piperidine), 1.46-1.49 (m, 2H, piperidine), 3.23-3.25 (m, 4H, piperidine), 3.33 (s, 2H, N*CH$_2$*CO), 4.11-4.16 (m, 1H, α-H), 4.24-4.30 (m, 1H, α-H), 4.56-4.63 (d, 4H, 2 x *CH$_2$*Ph), 6.38 (d,1H, NH), 7.14-7.37 (m, 13H, 2 x Ph and *CH*=CHCON and CH=*CH*CON and NH), 7.49 (s, 1H, NH), 8.05 (s,

1H, NH), 10.72 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{32}H_{42}N_7O_6$: 620.3191. Observed *m/z* 620.3143. Anal. Calcd. for $C_{32}H_{41}N_7O_6 \cdot 1.1H_2O$: C, 60.10; H, 6.81; N, 15.33. Found: C, 60.15; H, 6.71; N, 15.21.

N^2-(N-Piperidinocarbonylalanylalanyl)-N^1-*trans*-(3-benzyl-1-naphthylmethylcarbamoylpropenoyl)-N^1-carbamoylmethylhydrazine (27o, Pip-Ala-Ala-AAsn-CH=CH-CON(Bzl)-CH_2-1-naphthyl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (27% yield). ^{1}H-NMR (DMSO-d_6): 1.20 (d, 3H, Ala-CH_3), 1.27 (d, 3H, Ala-CH_3), 1.37 (s, 4H, piperidine), 1.48 (m, 2H, piperidine), 3.24 (s, 4H, piperidine), 3.33 (s, 2H, N*CH_2*CO), 4.13 (m, 1H, α-H), 4.28 (m, 1H, α-H), 4.65-4.67 (m, 2H, *CH_2*Ph), 5.06-5.17 (m, 2H, *CH_2*Naph) 6.39 (t, 1H, NH), 7.12-7.56 (m, 13H, Ph and Naph and *CH*=CHCON and CH=*CH*CON and 2 x NH) 7.84-8.05 (m, 4H, Naph and NH), 10.72 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{36}H_{44}N_7O_6$: 670.3348. Observed *m/z* 670.3374. Anal. Calcd. for $C_{36}H_{43}N_7O_6 \cdot 1.2H_2O$: C, 62.54; H, 6.62; N, 14.18. Found: C, 62.55; H, 6.61; N, 13.94.

N^2-(N-Piperidinocarbonylalanylalanyl)-N^1-carboxymethyl-N^1-trans-(3-di-1-Naphthylmethylcarbamoylpropenoyl)hydrazine (27p, Pip-Ala-Ala-AAsn-CH=CH-CON(CH_2-1-naphthyl)$_2$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (63% yield). ^{1}H NMR (DMSO-d_6): 1.20 (d, 3H, Ala-CH_3), 1.28 (d, 3H, Ala-CH_3), 1.35 (s, 4H, piperidine), 1.46 (m, 2H, piperidine), 3.23 (s, 4H, piperidine), 3.33 (s, 2H, N*CH_2*CO), 4.14 (m, 1H, α-H),

4.28 (m, 1H, α-H), 5.21 (s, 4H, 2 x N-*CH*$_2$-naphthyl), 6.38 (d, 1H, NH), 7.13-7.56 (m, 12H, naphthyl and CH=*CH*CON and *CH*=CHCON and 2 x NH), 7.82-8.10 (m, 7H, naphthyl and NH), 10.72 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{40}H_{46}N_7O_6$: 720.3568. Observed *m/z* 720.3504. Anal. Calcd. for $C_{40}H_{45}N_7O_6 \cdot 1.3H_2O$: C, 64.64; H, 6.45; N, 13.19. Found: C, 64.50; H, 6.32; N, 13.07.

N^2-(N-Morpholinocarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-ethoxycarbonylpropenoyl)hydrazine (28a, Mu-Ala-Ala-AAsn-CH=CH-COOEt). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (36% yield). ^{1}H-NMR (DMSO-d_6): 1.17-1.25 (m, 9H, 2 x Ala-CH_3 and $COCH_2CH_3$), 3.24-3.26 (m, 4H, morpholine), 3.32 (s, 2H, NCH_2CO), 3.50-3.52 (m, 4H, morpholine), 4.09-4.18 (m, 3H, α-H and $COCH_2CH_3$), 4.22 (m, 1H, α-H), 6.50 (d, 1H, NH), 6.57-6.61 (d, 1H, *CH*=CHCON), 7.17-7.20 (m, 2H, CH=*CH*CON and NH), 7.51 (s, 1H, NH), 8.08 (d, 1H, NH), 10.73 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{19}H_{31}N_6O_8$: 471.2227. Observed *m/z* 471.2198. Anal. Calcd. for $C_{19}H_{30}N_6O_8 \cdot 1H_2O$: C, 46.72; H, 6.60; N, 17.20. Found: C, 46.77; H, 6.62; N, 16.98.

N^2-(N-Morpholinocarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-phenylpropylcarbamoylpropenoyl)hydrazine (28h, Mu-Ala-Ala-AAsn-CH=CH-$CONHCH_2CH_2CH_2Ph$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (32% yield). ^{1}H-NMR (DMSO-d_6): 1.17-1.26 (d, 6H, 2 x Ala-CH_3), 1.68-1.75 (m, 2H, $NHCH_2CH_2CH_2Ph$), 2.55-2.59 (t, 2H, $NHCH_2CH_2CH_2Ph$), 3.11-3.16 (m, 2H, $NHCH_2CH_2CH_2Ph$), 3.25-3.29

(m, 4H, morpholine), 3.35 (s, 2H, N*CH*$_2$CO), 3.49-3.51 (m, 4H, morpholine), 4.10-4.17 (m, 1H, α-H), 4.22-4.28 (m, 1H, α-H), 6.53 (d, 1H, NH), 6.85-6.89 (d, 1H, *CH*=CHCON), 7.03-7.07 (d, 1H, CH=*CH*CON), 7.14-7.28 (m, 6H, Ph and NH), 7.50 (s, 1H, NH), 8.10 (s, 1H, NH), 8.49 (t, 1H, NH), 10.67 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{26}H_{38}N_7O_7$: 560.2834. Observed *m/z* 560.2817. Anal. Calcd. for $C_{26}H_{37}N_7O_7 \cdot 0.4H_2O$: C, 55.09; H, 6.72; N, 17.30. Found: C, 55.13; H, 6.71; N, 17.29.

N^2-(N-Morpholinocarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(3,4-dihydro-1H-isoquinolin-2-ylcarbonyl)propenoyl)hydrazine (28k, Mu-Ala-Ala-AAsn-CH=CHCO-tetrahydroisoquinoline). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (29% yield). ^{1}H-NMR (DMSO-d_6): 1.16-1.23 (m, 6H, 2 x Ala-CH_3), 2.79-2.85 (m, 2H, N-CH_2-*CH_2*-CH_2), 3.25-3.35 (m, 8H, morpholine and N*CH_2*CO and N-CH_2-CH_2-*CH_2*), 3.50 (m, 4H, morpholine), 3.72-3.75 (m, 2H, N-*CH_2*-CH_2), 4.12 (m, 1H, α-H), 4.25 (m, 1H, α-H), 4.65-4.75 (m, 2H, N-*CH_2*-C), 6.50 (d, 1H, NH), 7.04-7.42 (m, 7H, isoquinoline, *CH*=CHCON, CH=*CH*CON and NH), 7.53 (s, 1H, NH), 8.08 (d, 1H, NH), 10.68 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{26}H_{36}N_7O_7$: 558.2676. Observed *m/z* 558.2660. Anal. Calcd. for $C_{26}H_{35}N_7O_7 \cdot 1.8H_2O$: C, 52.93; H, 6.59; N, 16.62. Found: C, 53.00; H, 6.57; N, 16.67.

N^2-(N-Morpholinocarbonylalanylalanyl)-N^1-carboxymethyl-N^1-trans-(3-di-1-Naphthylmethylcarbamoylpropenoyl)hydrazine (28p, Mu-Ala-Ala-AAsn-CH=CH-CON(CH_2-1-naphthyl)$_2$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent.

Recrystallization with EtOAc/hexane gave a white solid. (40% yield). ^{1}H NMR (DMSO-d_6): 1.21 (d, 3H, Ala-CH_3), 1.29 (d, 3H, Ala-CH_3), 3.24 (m, 4H, morpholine), 3.29 (s, 2H, N*CH_2*CO), 3.48 (m, 4H, morpholine), 4.16 (m, 1H, α-H), 4.28 (m, 1H, α-H), 5.20 (s, 4H, 2 x N-CH_2-naphthyl), 6.52 (d, 1H, NH), 7.12-7.55 (m, 12H, naphthyl and CH=*CH*CON and *CH*=CHCON and NH_2), 7.82-8.10 (m, 7H, naphthyl and NH), 10.71 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{39}H_{44}N_7O_7$: 722.3340. Observed *m/z* 722.3297. Anal. Calcd. for $C_{39}H_{43}N_7O_7 \cdot 1.6H_2O$: C, 62.40; H, 6.20; N, 13.06. Found: C, 62.81; H, 6.23; N, 12.70.

N^2-(N-Piperazinocarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-ethoxycarbonylpropenoyl)hydrazine (37a, Piz-Ala-Ala-AAsn-CH=CH-COOEt). This compound was obtained by the removal of the *tert*-butyl protecting group of Boc-Piz-Ala-Ala-AAsn-CH=CH-COOEt with trifluoroacetic acid/methylene chloride (1:1) for 3 hours at room temperature. The volatiles were evaporated, and the TFA salt was washed several times with to give a white solid. (98% yield). ^{1}H-NMR (DMSO-d_6): 1.18-1.25 (m, 9H, 2 x Ala-CH_3 and $COCH_2CH_3$), 3.04 (s, 4H, piperazine), 3.36-3.50 (m, 6H, piperazine and N*CH_2*CO), 4.13-4.26 (m, 4H, 2 x α-H and CO*CH_2*CH_3), 6.57-6.61 (d, 1H, *CH*=CHCON), 6.75 (d, 1H, NH), 7.17-7.21 (m, 2H, CH=*CH*CON and NH), 7.53 (s, 1H, NH), 8.12 (d, 1H, NH), 8.78 (s, 1H, NH), 10.75 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{19}H_{32}N_7O_7$: 470.2358. Observed *m/z* 470.2386. Anal. Calcd. for $C_{19}H_{31}N_7O_7 \cdot 1.2TFA \cdot 1.5H_2O$: C, 40.58; H, 5.60; N, 15.48. Found: C, 40.85; H, 5.73; N, 15.31.

N^2-(N-Piperazinocarbonylalanylalanyl)-N^1-*trans*-(3-benzyloxycarbonylpropenoyl)-N^1-carbamoylmethylhydrazine (37b, Piz-Ala-Ala-

AAsn-CH=CH-COOBzl). This compound was obtained by the removal of the *tert*-butyl protecting group of Boc-Piz-Ala-Ala-AAsn-CH=CH-COOBzl with trifluoroacetic acid/methylene chloride (1:1) for 3 hours at room temperature. The volatiles were evaporated, and the TFA salt was washed several times with to give a white solid. (98% yield). ^{1}H-NMR (DMSO-d_6): 1.18 (s, 6H, 2 x Ala-CH_3), 3.03 (s, 4H, piperazine), 3.37-3.49 (m, 6H, piperazine and N*CH*$_2$CO), 4.12 (m, 1H, α-H), 4.22 (m, 1H, α-H), 5.19 (s, 2H, O*CH*$_2$Ph), 6.64-6.74 (m, 2H, *CH*=CHCON and NH), 7.20-7.37 (m, 7H, Ph and CH=*CH*CON and NH), 7.52 (s, 1H, NH), 8.11 (s, 1H, NH), 8.85 (s, 1H, NH), 10.76 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{24}H_{34}N_7O_7$: 532.2520. Observed *m/z* 532.2505. Anal. Calcd. for $C_{24}H_{33}N_7O_7 \cdot 1.05TFA \cdot 1.5H_2O$: C, 46.22; H, 5.51; N, 14.45. Found: C, 46.28; H, 5.49; N, 14.07.

N^2-(N-Piperazinocarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-dibenzylcarbamoylpropenoyl)hydrazine (37l, Piz-Ala-Ala-AAsn-CH=CH-CON(Bzl)$_2$). This compound was obtained by the removal of the *tert*-butyl protecting group of Boc-Piz-Ala-Ala-AAsn-CH=CH-CON(Bzl)$_2$ with trifluoroacetic acid/methylene chloride (1:1) for 3 hours at room temperature. The volatiles were evaporated, and the TFA salt was washed several times with to give a white solid. (99% yield). ^{1}H NMR (DMSO-d_6): 1.19-1.27 (m, 6H, 2 x Ala-CH_3), 3.03 (s, 4H, piperazine), 3.49 (m, 6H, piperazine and N*CH*$_2$CO), 4.14 (m, 1H, α-H), 4.26 (m, 1H, α-H), 4.56 (s, 2H, N*CH*$_2$Ph), 4.64 (s, 2H, N*CH*$_2$Ph), 6.76 (d, 1H, NH), 7.14-7.35 (m, 13H, 2 x Ph and CH=*CH*CON and *CH*=CHCON and NH), 7.50 (s, 1H, NH), 8.13 (s, 1H, NH), 8.81 (s, 1H, NH), 10.71 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{31}H_{41}N_8O_6$: 621.3179. Observed *m/z* 621.3144.

Anal. Calcd. for $C_{31}H_{40}N_8O_6 \cdot 1.7TFA \cdot 1.1H_2O$: C, 49.52; H, 5.30; N, 13.43. Found: C, 49.58; H, 5.64; N, 13.35.

N^2-(N-Piperazinocarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(methyl-1 naphthylmethylcarbamoyl)propenoyl)hydrazine (37n, Piz-Ala-Ala-AAsn-CH=CH-CON(CH_3)CH_2-1-naphthyl). This compound was obtained by the removal of the *tert*-butyl protecting group of Boc-Piz-Ala-Ala-AAsn-CH=CH-CON(CH_3)CH_2-1-naphthyl with trifluoroacetic acid/methylene chloride (1:1) for 3 hours at room temperature. The volatiles were evaporated, and the TFA salt was washed several times with to give a white solid. (99% yield). ^{1}H-NMR (DMSO-d_6): 1.19-1.28 (m, 6H, 2 x Ala-CH_3), 2.98-3.03 (m, 7H, piperazine and N-CH_3), 3.50-3.51 (m, 6H, piperazine and NCH_2CO), 4.13-4.29 (m, 2H, 2 x α-H), 5.04-5.09 (m, 2H, N-CH_2-naphthyl), 5.21 (s, 1H, NH), 6.76 (d, 1H, NH), 7.07-7.61 (m, 8H, naphthyl and CH=*CH*CON and *CH*=CHCON and 2 x NH), 7.86-8.13 (m, 3H, naphthyl), 8.83 (s, 1H, NH), 10.71 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{29}H_{39}N_8O_6$: 595.2987. Observed *m/z* 595.2947. Anal. Calcd. for $C_{29}H_{38}N_8O_6 \cdot 1.4TFA \cdot 2H_2O$: C, 48.33; H, 5.53; N, 14.18. Found: C, 48.38; H, 5.64; N, 13.88.

N^2-(N-Piperazinocarbonylalanylalanyl)-N^1-carboxymethyl-N^1-trans-(3-di-Naphthylmethylcarbamoylpropenoyl)hydrazine (37p, Piz-Ala-Ala-AAsn-CH=CH-CON(CH_2-1-naphthyl)$_2$). This compound was obtained by the removal of the *tert*-butyl protecting group of Boc-Piz-Ala-Ala-AAsn-CH=CH-CON(CH_2-1-naphthyl)$_2$ with trifluoroacetic acid/methylene chloride (1:1) for 3 hours at room temperature. The volatiles were evaporated, and the TFA salt was washed several times with to give a white solid. (93% yield). ^{1}H NMR (DMSO-d_6): 1.21 (d, 3H, Ala-CH_3), 1.29 (d, 3H, Ala-

CH_3), 1.37 (s, 9H, Boc), 3.03 (s, 4H, piperazine), 3.50-3.73 (m, 6H, piperazine and N*CH*$_2$CO), 4.16 (m, 1H, α-H), 4.28 (m, 1H, α-H), 5.21 (s, 4H, 2 x N-CH_2-naphthyl), 6.76 (d, 1H, NH), 7.13-7.56 (m, 12H, naphthyl and CH=*CH*CON and *CH*=CHCON and 2 x NH), 7.82-8.14 (m, 7H, naphthyl and NH), 8.82 (s, 1H, NH), 10.73 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{39}H_{45}N_8O_6$: 721.3384. Observed *m/z* 721.3379. Anal. Calcd. for $C_{39}H_{44}N_8O_6 \cdot 1.6TFA \cdot 1H_2O$: C, 55.02; H, 5.21; N, 12.16. Found: C, 54.83; H, 5.34; N, 12.07.

N^2-(4-(*tert*-butoxycarbonyl)piperazin-1-ylcarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-ethoxycarbonylpropenoyl)hydrazine (29a, Boc-Piz-Ala-Ala-AAsn-CH=CH-COOEt). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (34% yield). ^{1}H-NMR (DMSO-d_6): 1.17-1.25 (m, 9H, 2 x Ala-CH_3 and $COCH_2CH_3$), 1.38 (s, 9H, Boc), 3.26 (s, 8H, piperazine), 3.32 (s, 2H, N*CH*$_2$CO), 4.08-4.26 (m, 4H, 2 x α-H and CO*CH*$_2$CH$_3$), 6.53-6.61 (m, 2H, *CH*=CHCON and NH), 7.17-7.20 (m, 2H, CH=*CH*CON and NH), 7.51 (s, 1H, NH), 8.09 (d, 1H, NH), 10.72 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{24}H_{40}N_7O_9$: 570.2882. Observed *m/z* 570.2906. Anal. Calcd. for $C_{24}H_{39}N_7O_9 \cdot 0.9H_2O$: C, 49.21; H, 7.02; N, 16.74. Found: C, 49.28; H, 7.03; N, 16.62.

N^2-(4-(*tert*-butoxycarbonyl)piperazin-1-ylcarbonylalanylalanyl)-N^1-*trans*-(3-benzyloxycarbonylpropenoyl)-N^1-carbamoylmethylhydrazine (29b, Boc-Piz-Ala-Ala-AAsn-CH=CH-COOBzl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (25% yield). ^{1}H-

NMR (DMSO-d_6): 1.16-1.21 (m, 6H, 2 x CH_3), 1.38 (s, 9H, Boc), 3.25 (s, 8H, piperazine), 3.33 (s, 2H, N*CH_2*CO), 4.11 (m, 1H, α-H), 4.21 (m, 1H, α-H), 5.19 (s, 2H, O-CH_2-Ph), 6.54 (d, 1H, NH), 6.63-6.67 (d, 1H, *CH*=CHCON), 7.22-7.39 (m, 7H, Ph and CH=*CH*CON and NH), 7.52 (s, 1H, NH), 8.09 (s, 1H, NH), 10.45 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{29}H_{42}N_7O_9$: 632.6771. Observed *m/z* 632.6753. Anal. Calcd. for $C_{29}H_{41}N_7O_9 \cdot 0.9H_2O$: C, 53.76; H, 6.66; N, 15.13. Found: C, 53.76; H, 6.66; N, 14.91.

N^2-(4-(*tert*-butoxycarbonyl)piperazin-1-ylcarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-dibenzylcarbamoylpropenoyl)hydrazine (29l, Boc-Piz-Ala-Ala-AAsn-CH=CH-CON(Bzl)$_2$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (50% yield). ^{1}H NMR (DMSO-d_6): 1.19 (d, 3H, CH_3), 1.26 (d, 3H, CH_3), 1.38 (s, 9H, Boc), 3.25 (s, 8H, piperazine), 3.32 (s, 2H, N*CH_2*CO), 4.14 (m, 1H, α-H), 4.26 (m, 1H, α-H), 4.56 (s, 2H, N-CH_2-Ph), 4.63 (s, 2H, N-CH_2-Ph), 6.55 (d, 1H, NH), 7.14-7.37 (m, 13H, 2 x Ph and CH=*CH*CON and *CH*=CHCON and NH), 7.82-8.10 (m, 7H, naphthyl and NH), 7.48 (s, 1H, NH), 8.09 (d, 1H, NH), 10.69 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{36}H_{49}N_8O_8$: 721.3668. Observed *m/z* 721.3660. Anal. Calcd. for $C_{36}H_{48}N_8O_8 \cdot 1.5H_2O$: C, 57.82; H, 6.87; N, 14.98. Found: C, 57.94; H, 6.84; N, 14.93.

N^2-(4-(*tert*-butoxycarbonyl)piperazin-1-ylcarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(methyl-1 naphthylmethylcarbamoyl)propenoyl)hydrazine (29n, Boc-Piz-Ala-Ala-AAsn-CH=CH-CON(CH_3)CH_2-1-naphthyl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10%

MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (29% yield). ^{1}H-NMR (DMSO-d_6): 1.18-1.26 (m, 6H, 2 x CH_3), 1.37 (s, 9H, Boc), 2.99 (d, 3H, N-CH_3), 3.26 (s, 8H, piperazine), 3.34 (s, 2H, N*CH_2*CO), 4.14 (m, 1H, α-H), 4.28 (m, 1H, α-H), 5.04 (m, 2H, N-CH_2-naphthyl), 5.21 (s, 1H, NH), 6.58 (d, 1H, NH), 7.07-7.61 (m, 8H, naphthyl and CH=*CH*CON and *CH*=CHCON and NH_2), 7.85-8.10 (m, 3H, naphthyl), 8.12 (d, 1H, NH), 10.42 (s, 1H, NH), 10.72 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{34}H_{47}N_8O_8$: 695.3511. Observed *m/z* 695.3559. Anal. Calcd. for $C_{34}H_{46}N_8O_8 \cdot 1.4H_2O$: C, 57.00; H, 6.81; N, 15.64. Found: C, 57.05; H, 6.81; N, 15.44.

N^2-(4-(*tert*-butoxycarbonyl)piperazin-1-ylcarbonylalanylalanyl)-N^1-carboxymethyl-N^1-trans-(3-di-1-Naphthylmethylcarbamoylpropenoyl)hydrazine (29p, Boc-Piz-Ala-Ala-AAsn-CH=CH-CON(CH_2-1-naphthyl)$_2$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (25% yield). ^{1}H NMR (DMSO-d_6): 1.20 (d, 3H, CH_3), 1.28 (d, 3H, CH_3), 1.37 (s, 9H, Boc), 3.25 (s, 8H, piperazine), 3.33 (s, 2H, N*CH_2*CO), 4.15 (m, 1H, α-H), 4.25 (m, 1H, α-H), 5.20 (s, 4H, 2 x N-CH_2-naphthyl), 6.57 (d, 1H, NH), 7.11-7.56 (m, 12H, naphthyl and CH=*CH*CON and *CH*=CHCON and NH_2), 7.82-8.10 (m, 7H, naphthyl and NH), 10.43 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{44}H_{53}N_8O_8$: 821.4025. Observed *m/z* 821.3981. Anal. Calcd. for $C_{44}H_{52}N_8O_8 \cdot 1.1H_2O$: C, 62.86; H, 6.50; N, 13.33. Found: C, 62.87; H, 6.41; N, 13.14.

N^2-(4-(Benzyloxycarbonyl)piperazin-1-ylcarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-trans-(3-ethoxycarbonylpropenoyl)hydrazine (30a, Cbz-Piz-Ala-Ala-AAsn-CH=CH-COOEt). This compound was obtained using the HOBt/EDC

coupling method and purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (41% yield). ^{1}H NMR (DMSO-d_6): 1.17-1.25 (m, 9H, 2 x Ala-CH_3 and $COCH_2CH_3$), 3.31-3.34 (m, 10H, piperazine and NCH_2CO), 4.09-4.18 (m, 3H, α-H and $COCH_2CH_3$), 4.24 (q, 1H, α-H), 5.07 (s, 2H, Cbz), 6.55-6.61 (m, 2H, NH and *CH*=CHCON), 7.18-7.38 (m, 7H, Ph and NH and CH=*CH*CON), 7.50 (s, 1H, NH), 8.09 (d, 1H, NH), 10.72 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{27}H_{38}N_7O_9$: 604.2726. Observed *m/z* 604.2777. Anal. Calcd. for $C_{27}H_{37}N_7O_9 \cdot 0.9H_2O$: C, 52.32; H, 6.31; N, 15.82. Found: C, 52.36; H, 6.33; N, 15.73.

N^2-(4-(Benzyloxycarbonyl)piperazin-1-ylcarbonylalanylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(methyl-1-naphthylmethylcarbamoyl)propenoyl)hydrazine (30n, Cbz-Piz-Ala-Ala-AAsn-CH=CH-CON(CH_3)CH_2-1-naphthyl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (38% yield). 1.15-1.28 (m, 6H, 2 x CH_3), 2.99 (d, 3H, N-CH_3), 3.32 (s, 8H, piperazine), 3.34 (s, 2H, NCH_2CO), 4.14 (m, 1H, α-H), 4.26 (m, 1H, α-H), 5.06 (m, 4H, Cbz and N-CH_2-naphthyl), 5.20 (s, 1H, NH), 6.60 (t, 1H, NH), 7.07-7.61 (m, 12H, naphthyl and CH=*CH*CON and *CH*=CHCON and NH_2), 7.85-8.10 (m, 4H, naphthyl), 10.43 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{37}H_{45}N_8O_8$: 729.3355. Observed *m/z* 729.3376. Anal. Calcd. for $C_{37}H_{44}N_8O_8 \cdot 1.3H_2O$: C, 59.08; H, 6.24; N, 14.90. Found: C, 59.17; H, 6.27; N, 14.56.

N^2-(4-(Benzyloxycarbonyl)piperazin-1-ylcarbonylalanylalanyl)-N^1-carboxymethyl-N^1-trans-(3-di-1-naphthylmethylcarbamoylpropenoyl)hydrazine

(30p, Cbz-Piz-Ala-Ala-AAsn-CH=CH-CON(CH_2-1-naphthyl)$_2$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (39% yield). 1.20 (d, 3H, Ala-CH_3), 1.28 (d, 3H, Ala-CH_3), 3.31 (m, 8H, piperazine), 3.34 (s, 2H, N*CH_2*CO), 4.16 (m, 1H, α-H), 4.27 (m, 1H, α-H), 5.06 (s, 2H, Cbz), 5.20 (s, 4H, N-CH_2-Ph and N-CH_2-naphthyl), 6.59 (d, 1H, NH), 7.12-7.54 (m, 17H, naphthyl and Ph and CH=*CH*CON and *CH*=CHCON and NH_2), 7.82-8.13 (m, 5H, naphthyl and NH), 10.44 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{47}H_{51}N_8O_8$: 855.3838. Observed *m/z* 855.3824. Anal. Calcd. for $C_{47}H_{50}N_8O_8 \cdot 1.2H_2O$: C, 64.40; H, 6.03; N, 12.78. Found: C, 64.42; H, 5.96; N, 12.57.

N^2-(N-Benzyloxycarbonylalanylvalyl)-N^1-carbamoylmethyl-N^1-trans-(3-ethoxycarbonylpropenoyl)hydrazine (31a, Cbz-Ala-Val-AAsn-CH=CH-COOEt). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (25% yield). ^{1}H NMR (DMSO-d_6): 0.84-0.85 (d, 6H, 2 x Val-CH_3), 1.15-1.21 (m, 6H, Ala-CH_3 and COCH$_2$*CH_3*), 1.97 (s, 1H, CH), 3.32 (s, 2H, NH*CH_2*CO), 4.08-4.17 (m, 4H, 2 x α-H and CO*CH_2*CH_3), 4.95-5.02 (m, 2H, Cbz), 6.57-6.61 (d, 1H, *CH*=CHCON), 7.18-7.44 (m, 7H, Ph and CH=*CH*CON and NH), 7.50 (s, 1H, NH), 7.92 (s, 1H, NH), 10.90 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{24}H_{34}N_5O_8$: 520.2456. Observed *m/z* 520.2402. Anal. Calcd. for $C_{24}H_{33}N_5O_8 \cdot 1.9CH_2Cl_2$: C, 45.69; H, 5.45; N, 10.29. Found: C, 45.87; H, 5.35; N, 10.14.

N^2-(N-Benzyloxycarbonylalanylvalyl)-N^1-carbamoylmethyl-N^1-trans-(3-1-naphthylmethylcarbamoylpropenoyl)hydrazine (31m, Cbz-Ala-Val-AAsn-CH=CH-

CONHCH$_2$-1-naphthyl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (11% yield). ^{1}H NMR (DMSO-d_6): 0.85 (s, 6H, 2 x Val-CH_3), 1.17 (d, 3H, Ala-CH_3), 2.00 (s, 1H, CH), 3.32 (s, 2H, NH*CH_2*CO), 4.11-4.22 (m, 2H, 2 x α-H), 4.81 (d, 2H, NH*CH_2*-Naph), 4.99 (s, 2H, Cbz), 6.91-6.95 (d, 1H, *CH*=CHCON), 7.13-7.54 (m, 12H, Ph and Naph and CH=*CH*CON and NH), 7.73-8.02 (m, 3H, Naph and NH), 8.96 (s, 1H, NH), 10.85 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{33}H_{39}N_6O_7$: 631.2875. Observed *m/z* 631.2885. Anal. Calcd. for $C_{33}H_{38}N_6O_7 \cdot 1.4H_2O$: C, 60.43; H, 6.27; N, 12.81. Found: C, 60.46; H, 5.96; N, 12.71.

N^2-(N-Benzyloxycarbonylalanylvalyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(3,4-dihydro-2H-quinolin-1-ylcarbonyl)propenoyl)hydrazine (31j, Cbz-Ala-Val-AAsn-CH=CH-CO-tetrahydroquinoline). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a yellowish solid (36% yield). ^{1}H NMR (DMSO-d_6): 0.86-0.88 (d, 6H, 2 x CH_3), 1.79 (d, 3H, Ala-CH_3), 1.83-1.89 (m, 2H, N-CH_2-*CH_2*-CH_2), 2.00 (s, 1H, CH), 2.67-2.70 (t, 2H, N-CH_2-CH_2-*CH_2*), 3.32 (s, 2H, NH*CH_2*CO), 3.70-3.73 (m, 2H, N-*CH_2*-CH_2-CH_2), 4.12 (m, 1H, α-H), 4.23 (m, 1H, α-H), 4.96-5.03 (m, 2H, Cbz), 7.07-7.44 (m, 13H, Ph, quinoline, *CH*=CHCON, CH=*CH*CON and NH), 7.49 (s, 1H, NH), 7.93 (s, 1H, NH), 10.88 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{31}H_{39}N_6O_7$: 607.2875. Observed *m/z* 607.2910. Anal. Calcd. for $C_{31}H_{38}N_6O_7 \cdot 0.3H_2O$: C, 60.73; H, 6.51; N, 13.71. Found: C, 60.71; H, 6.27; N, 13.58.

N^2-(N-Benzyloxycarbonylalanylisoleucyl)-N^1-carbamoylmethyl-N^1-trans-(3-ethoxycarbonylpropenoyl)hydrazine (32a, Cbz-Ala-Ile-AAsn-CH=CH-COOEt). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white solid (23% yield). ^{1}H NMR (DMSO-d_6): 0.79-0.82 (m, 6H, 2 x Ile-CH_3), 1.15-1.24 (m, 7H, CH and Ala-CH_3 and $COCH_2\mathit{CH_3}$), 1.43 (m, 1H, CH), 1.73 (m, 1H, CH), 3.32 (s, 2H, $NH\mathit{CH_2}CO$), 4.06-4.24 (m, 4H, 2 x α-H and $CO\mathit{CH_2}CH_3$), 4.99 (s, 2H, Cbz), 6.58-6.62 (d, 1H, *CH*=CHCON), 7.19-7.36 (m, 7H, Ph and CH=*CH*CON and NH), 7.49 (s, 1H, NH), 7.94 (s, 1H, NH), 10.90 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{25}H_{36}N_5O_8$: 534.2558. Observed *m/z* 534.2600.

N^2-(N-Benzyloxycarbonylalanylphenylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-3-(2-furyl)carbamoylpropenoyl)hydrazine (33e, Cbz-Ala-Phe-AAsn-CH=CH-$CONHCH_2$-2-furyl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a yellowish solid (7% yield). ^{1}H NMR (DMSO-d_6): 1.11 (d, 3H, Ala-CH_3), 2.81-2.98 (m, 2H, $\mathit{CH_2}$Ph), 3.32 (d, 2H, $NH\mathit{CH_2}CO$) 4.01 (m, 1H, α-H), 4.34 (d, 2H, $\mathit{CH_2}$-furyl), 4.53 (m, 1H, α-H), 4.96-4.99 (m, 2H, Cbz), 6.24 (d, 1H, furyl CH), 6.36 (t, 1H, furyl CH), 6.87-6.91 (d, 1H, *CH*=CHCON), 7.09-7.13 (d, 1H, CH=*CH*CON), 7.18-7.34 (m, 11H, 2 x Ph and NH), 7.43 (s, 1H, NH), 7.55 (d, 1H, furyl CH), 8.19 (s, 1H, NH), 8.91 (t, 1H, NH), 10.89 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{31}H_{35}N_6O_8$: 619.2511. Observed *m/z* 619.2575. Anal. Calcd. for $C_{31}H_{34}N_6O_8 \cdot 0.75H_2O$: C, 58.90; H, 5.66; N, 13.29. Found: C, 58.95; H, 5.52; N, 13.10.

N^2-(N-Benzyloxycarbonylalanylphenylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(3,4-dihydro-2H-quinolin-1-ylcarbonyl)propenoyl)hydrazine (33j, Cbz-Ala-Phe-AAsn-CH=CH-CO-tetrahydroquinoline). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a yellowish solid (27% yield). ^{1}H NMR (DMSO-d_6): 1.12 (d, 3H, Ala-CH_3), 1.84-1.89 (m, 2H, N-CH_2-*CH_2*-CH_2), 2.67-2.70 (t, 2H, NCH_2CH_2*CH_2*), 2.87-3.01 (m, 2H, *CH_2*Ph), 3.32 (s, 2H, NH*CH_2*CO), 3.70-3.71 (m, 2H, N*CH_2*CH_2CH_2), 4.03 (m, 1H, α-H), 4.58 (m, 1H, α-H), 4.94-5.02 (m, 2H, Cbz), 7.05-7.34 (m, 17H, 2 x Ph, quinoline, *CH*=CHCON, CH=*CH*CON and NH), 7.48 (s, 1H, NH), 8.18 (s, 1H, NH), 10.92 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{35}H_{39}N_6O_7$: 655.2875. Observed *m/z* 655.2923. Anal. Calcd. for $C_{35}H_{38}N_6O_7 \cdot 0.4H_2O$: C, 63.51; H, 5.91; N, 12.70. Found: C, 63.47; H, 5.84; N, 12.67.

N^2-(N-Benzyloxycarbonylalanylphenylalanyl)-N^1-carbamoylmethyl-N^1-*trans*-(3-(3,4-dihydro-2H-isoquinolin-1-ylcarbonyl)propenoyl)hydrazine (33k, Cbz-Ala-Phe-AAsn-CH=CH-CO-tetrahydroisoquinoline). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (11% yield). ^{1}H NMR (DMSO-d_6): 1.10 (d, 3H, Ala-CH_3), 2.76-2.98 (m, 4H, *CH_2*Ph and N-CH_2-CH_2-*CH_2*), 3.35 (s, 2H, NH*CH_2*CO), 3.72 (m, 2H, N-*CH_2*-CH_2), 4.02 (m, 1H, α-H), 4.56 (m, 1H, α-H), 4.65-4.73 (m, 2H, N-*CH_2*-C), 4.94-5.02 (m, 2H, Cbz), 7.00-7.33 (m, 12H, Ph, isoquinoline, *CH*=CHCON, CH=*CH*CON and NH), 7.51 (s, 1H, NH), 8.18 (s, 1H, NH), 10.89 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{35}H_{39}N_6O_7$: 655.2875.

Observed *m/z* 655.2893. Anal. Calcd. for $C_{35}H_{38}N_6O_7 \cdot 0.75EtOAc$: C, 63.33; H, 6.15; N, 11.67. Found: C, 63.45; H, 5.99; N, 11.68.

(*2S,3S*)-3-(N^2-(N-Piperidinocarbonylalanylalanyl)-N^1-carbamoylmethylhydrazinocarbonyl)oxirane-2-carboxylic Acid Ethyl Ester (34q, Pip-Ala-Ala-AAsn-EP(*S,S*)-COOEt). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% $MeOH/CH_2Cl_2$ as the eluent. Recrystallization with EtOAc/hexane gave a white powder (3% yield). ^{1}H-NMR (DMSO-d_6): 1.12-1.20 (m, 9H, 2 x Ala-CH_3 and $COCH_2CH_3$), 1.39 (m, 4H, piperidine), 1.49 (m, 2H, piperidine), 3.25 (m, 4H, piperidine), 3.32 (s, 2H, NCH_2CO), 3.49 (s, 1H, epoxy CH), 3.67 (s, 1H, epoxy CH), 4.05-4.28 (m, 4H, 2 x α-H and $COCH_2CH_3$), 6.41 (d, 1H, NH), 7.23 (s, 1H, NH) 7.51 (s, 1H, NH) 8.06 (d, 1H, NH), 10.72 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{20}H_{33}N_6O_8$: 485.2354. Observed *m/z* 485.2363. Anal. Calcd. for $C_{20}H_{32}N_6O_8 \cdot 1.2H_2O$: C, 47.46; H, 6.85; N, 16.61. Found: C, 47.47; H, 6.57; N, 16.92.

(*2S,3S*)-2-(di-Benzylcarbamoyl)-3-(N^2-(N-Piperidinocarbonylalanylalanyl)-N^1-carbamoylmethylhydrazinocarbonyl)oxirane (34r, Pip-Ala-Ala-AAsn-EP(*S,S*)-CON(Bzl)$_2$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% $MeOH/CH_2Cl_2$ as the eluent. Recrystallization with EtOAc/hexane gave a white powder (29% yield). ^{1}H-NMR (DMSO-d_6): 1.17-1.19 (m, 6H, 2 x Ala-CH_3), 1.39 (m, 4H, piperidine), 1.49 (m, 2H, piperidine), 3.26 (m, 4H, piperidine), 3.32 (s, 2H, NCH_2CO), 3.80 (s, 1H, epoxy CH), 4.05-4.12 (m, 1H, α-H), 4.23-4.74 (m, 6H, α-H and epoxy CH and 2 x N-CH_2-Ph), 6.41 (d, 1H, NH), 7.21-7.39 (m, 11H, 2 x Ph and NH) 7.47 (s, 1H, NH) 8.09 (s, 1H, NH),

10.68 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{32}H_{42}N_7O_7$: 636.3140. Observed *m/z* 636.3129. Anal. Calcd. for $C_{32}H_{41}N_7O_7 \cdot 1.1H_2O$: C, 58.63; H, 6.64; N, 14.96. Found: C, 58.58; H, 6.60; N, 14.92.

(*2S,3S*)-2-(1-Naphthylmethyl arbamoyl)-3-(N^2-(N-Piperidinocarbonylalanylalanyl)-N^1-carbamoylmethylhydrazinocarbonyl)oxirane (34s, Pip-Ala-Ala-AAsn-EP(*S,S*)-CONHCH$_2$-1-naphthyl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a yellowish solid (47% yield). ^{1}H NMR (DMSO-d_6): 1.14-1.20 (m, 6H, 2 x Ala-CH_3), 1.38 (m, 4H, piperidine), 1.48 (m, 2H, piperidine), 3.25 (m, 4H, piperidine), 3.32 (s, 2H, N*CH$_2$*CO), 3.52 (s, 1H, epoxy CH), 4.10-4.20 (m, 3H, 2 x α-H and epoxy CH), 4.73-4.78 (m, 2H, N-*CH$_2$*-naphthyl), 6.36 (d, 1H, NH), 7.21 (s, 1H, NH), 7.43-7.58 (m, 4H, naphthyl), 7.45-7.56 (m, 4H, naphthyl), 7.84-8.03 (m, 4H, naphthyl and NH), 9.00 (s, 1H, NH), 10.77 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{29}H_{38}N_7O_7$: 596.2827. Observed *m/z* 596.2847. Anal. Calcd. for $C_{29}H_{37}N_7O_7 \cdot 1.4H_2O$: C, 56.10; H, 6.46; N, 15.79. Found: C, 56.06; H, 6.25; N, 15.77.

(*2S,3S*)-2-(1-Naphthylmethylcarbamoyl)-3-(N^2-(N-Morpholinocarbonylalanylalanyl)-N^1-carbamoylmethylhydrazinocarbonyl)oxirane (35s, Mu-Ala-Ala-AAsn-EP(*S,S*)-CONHCH$_2$-1-naphthyl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (22% yield). ^{1}H NMR (DMSO-d_6): 1.15-1.21 (m, 6H, 2 x Ala-CH_3), 3.26 (m, 4H, morpholine), 3.34 (s, 2H, N*CH$_2$*CO), 3.50 (m, 4H, morpholine), 4.12-4.20 (m,

4H, 2 x α-H and 2 x epoxy CH), 4.28 (m, 1H, α-H), 4.70-4.82 (m, 2H, N-*CH*$_2$-naphthyl), 6.52 (d, 1H, NH), 7.21 (s, 1H, NH), 7.43-7.58 (m, 4H, naphthyl), 7.84-8.10 (m, 4H, naphthyl and NH), 9.00 (s, 1H, NH), 10.77 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{28}H_{36}N_7O_8$: 598.2620. Observed *m/z* 598.2656. Anal. Calcd. for $C_{28}H_{35}N_7O_8 \cdot 1.7H_2O$: C, 53.53; H, 6.16; N, 15.61. Found: C, 53.41; H, 6.14; N, 15.63.

(*2S,3S*)-2-(di-Benzylcarbamoyl)-3-(N^2-(N-Morpholinocarbonylalanylalanyl)-N^1-carbamoylmethylhydrazinocarbonyl)oxirane (35r, Mu-Ala-Ala-AAsn-EP(*S,S*)-CON(Bzl)$_2$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (19% yield). ^{1}H-NMR (DMSO-d_6): 1.18 (m, 6H, 2 x Ala-CH_3), 3.27 (m, 4H, morpholine), 3.34 (s, 2H, N*CH*$_2$CO), 3.50 (m, 4H, morpholine), 3.80-4.25 (m, 4H, 2 x α-H, 2 x epoxy CH), 4.55-4.78 (m, 4H, 2 x N-CH_2-Ph), 6.55 (d, 1H, NH), 7.22-7.47 (m, 12H, 2 x Ph and 2 x NH), 8.15 (d, 1H, NH), 10.66 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{31}H_{40}N_7O_8$: 638.2938. Observed *m/z* 638.2937. Anal. Calcd. for $C_{31}H_{39}N_7O_8 \cdot 1.4H_2O$: C, 56.17; H, 6.36; N, 14.79. Found: C, 56.12; H, 6.34; N, 14.76.

(*cis*)-2-(di-Benzylcarbamoyl)-3-(N^2-(N-Morpholinocarbonylalanylalanyl)-N^1-carbamoylmethylhydrazinocarbonyl)oxirane (35t, Mu-Ala-Ala-AAsn-EP(*cis*)-CON(Bzl)$_2$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (13% yield). ^{1}H-NMR (DMSO-d_6): 1.16-1.25 (m, 6H, 2 x Ala-CH_3), 3.24 (m, 4H, morpholine), 3.33 (s, 2H, N*CH*$_2$CO), 3.49 (m, 4H, morpholine), 4.04-4.25 (m, 4H, 2 x α-H, 2 x epoxy CH), 4.37-

4.73 (m, 4H, 2 x N-CH_2-Ph), 6.53 (d, 1H, NH), 7.15-7.41 (m, 12H, 2 x Ph and 2 x NH), 8.16 (d, 1H, NH), 10.87 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{31}H_{40}N_7O_8$: 638.2938. Observed *m/z* 638.2943. Anal. Calcd. for $C_{31}H_{39}N_7O_8 \cdot 1.5H_2O$: C, 56.02; H, 6.37; N, 14.75 Found: C, 56.00; H, 6.41; N, 14.69.

(*2S,3S*)-2-(di-Benzylcarbamoyl)-3-(N^2-(N-Piperazinocarbonylalanylalanyl)-N^1-carbamoylmethylhydrazinocarbonyl)oxirane (38r, Piz-Ala-Ala-AAsn-EP(*S,S*)-CON(Bzl)$_2$). This compound was obtained by the removal of the *tert*-butyl protecting group of Boc-Piz-Ala-Ala-AAsn-EP(*S,S*)-CON(Bzl)$_2$ with trifluoroacetic acid/methylene chloride (1:1) for 3 hours at room temperature. The volatiles were evaporated, and the TFA salt was washed several times with to give a white solid. (91% yield). ^{1}H NMR (DMSO-d_6): 1.18-1.21 (m, 6H, 2 x Ala-CH_3), 3.03 (s, 4H, piperazine), 3.37-3.51 (m, 6H, piperazine and N*CH_2*CO), 3.83 (s, 1H, epoxide CH), 4.10-4.33 (m, 3H, 2 x α-H and epoxide CH), 4.53-4.57 (d, 4H, 2 x N*CH_2*Ph), 6.76 (d, 1H, NH), 7.21-7.39 (m, 11H, 2 x Ph and NH), 7.47 (s, 1H, NH), 8.18 (s, 1H, NH), 8.71 (s, 1H, NH), 10.62 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{31}H_{41}N_8O_7$: 637.3093. Observed *m/z* 637.3032. Anal. Calcd. for $C_{31}H_{40}N_8O_7 \cdot 1.1TFA \cdot 2H_2O$: C, 49.96; H, 5.70; N, 14.04. Found: C, 50.36; H, 5.75; N, 13.44.

(*2S,3S*)-2-(di-Benzylcarbamoyl)-3-(N^2-(4-(*tert*-butoxycarbonyl)piperazin-1-yl carbonylalanylalanyl)-N^1-carbamoylmethylhydrazinocarbonyl)oxirane (36r, Boc-Piz-Ala-Ala-AAsn-EP(*S,S*)-CON(Bzl)$_2$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (22% yield). ^{1}H NMR (DMSO-d_6): 1.18 (s, 6H, 2 x Ala-CH_3), 1.38 (s, 9H, Boc), 3.25-

3.31 (m, 10H, piperazine and N*CH*$_2$CO), 3.81 (s, 1H, epoxide CH), 4.08-4.30 (m, 3H, 2 x α-H and epoxide CH), 4.57 (s, 2H, N*CH*$_2$Ph), 4.70 (s, 2H, N*CH*$_2$Ph), 6.57 (d, 1H, NH), 7.17-7.36 (m, 11H, 2 x Ph and NH), 7.46 (s, 1H, NH), 8.14 (s, 1H, NH), 10.67 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{36}H_{49}N_8O_9$: 737.3617. Observed *m/z* 737.3630. Anal. Calcd. for $C_{36}H_{48}N_8O_9 \cdot 1.4H_2O$: C, 56.74; H, 6.72; N, 14.27. Found: C, 56.66; H, 6.71; N, 14.70

(39, Mu-Ala-AAsn-CO-CH$_2$CH$_2$Ph). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (60% yield). ^{1}H NMR (DMSO-d$_6$): 1.20 (d, 3H, Ala-CH_3), 2.54-2.58 (m, 2H, *CH*$_2$CH$_2$Ph), 2.73-2.76 (t, 2H, CH$_2$*CH*$_2$Ph), 3.18-3.26 (m, 4H, morpholine), 3.34 (d, 2H, N*CH*$_2$CO), 3.46-3.48 (m, 4H, morpholine), 4.06-4.13 (m, 1H, α-H), 6.68 (d, 1H, NH), 7.14-7.25 (m, 6H, Ph and NH), 7.53 (s, 1H, NH), 10.38 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{19}H_{28}N_5O_5$: 406.2085. Observed *m/z* 406.2093. Anal. Calcd. for $C_{19}H_{27}N_5O_5 \cdot 0.7H_2O$: C, 54.59; H, 6.85; N, 16.75. Found: C, 54.82; H, 6.83; N, 16.82.

(40, Pip-Ala-AAsn-CONHPh-2-OPh). 2-phenoxybenzeneisocyanate (1 eq) was dissolved in CH_2Cl_2 and cooled down to 0 °C. Then peptidyl hydrazide (1 eq) was added and reaction mixture was stirred at room temperature overnight. The solvent was removed and compound was purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (62% yield). ^{1}H NMR (DMSO-d$_6$): 1.12 (d, 3H, Ala-CH_3), 1.34-1.37 (m, 4H, piperidine), 1.45-1.49 (m, 2H, piperidine), 3.15 (d, 2H, N*CH*$_2$CO), 3.17-3.22 (m, 4H, piperidine), 3.95 (m, 1H, α-H), 6.58 (d, 1H, NH), 6.78 (d, 1H, CH), 6.98-7.15 (m, 7H, 2 x

Ph and NH), 7.34-7.38 (m, 2H, Ph), 7.56 (s, 1H, NH), 10.23 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{24}H_{31}N_6O_5$: 483.2350. Observed *m/z* 483.2313. Anal. Calcd. for $C_{24}H_{30}N_6O_5 \cdot 0.05H_2O$: C, 59.63; H, 6.28; N, 17.38. Found: C, 59.65; H, 6.25; N, 17.34.

(41, Mu-Ala-AAsn-CONH-1-naphthyl). This compound was obtained from the reaction of 1-naphthaleneisocyanate with Mu-Ala-AAsn and purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (72% yield). ^{1}H NMR (DMSO-d_6): 1.29 (d, 3H, Ala-CH_3), 3.15 (d, 2H, N*CH_2*CO), 3.31-3.34 (m, 8H, morpholine), 4.04 (m, 1H, NH), 4.09 (m, 1H, α-H), 6.99 (d, 1H, NH), 7.21 (s, 1H, NH), 7.38 (d, 1H, CH), 7.43-7.51 (m, 3H, 3 x CH), 7.72-7.75 (m, 2H, NH and CH), 7.88 (d, 1H, CH), 7.96 (d, 1H, CH), 8.83 (s, 1H, NH), 10.59 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{21}H_{27}N_6O_5$: 443.2043. Observed *m/z* 443.2064. Anal. Calcd. for $C_{21}H_{26}N_6O_5 \cdot 2.5H_2O$: C, 51.74; H, 6.14; N, 17.24. Found: C, 51.78; H, 6.15; N, 16.92.

(42, Cbz-Piz-Ala-AAsn-CONHCH$_2$Ph). This compound was obtained from the reaction of benzylisocyanate with Cbz-Piz-Ala-AAsn and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (71% yield). ^{1}H NMR (DMSO-d_6): 1.23 (d, 3H, Ala-CH_3), 3.17-3.24 (m, 8H, piperazine), 3.36 (s, 2H, N*CH_2*CO), 3.84-3.95 (m, 2H, *CH_2*Ph), 4.18-4.26 (m, 1H, α-H), 5.07 (s, 2H, Cbz), 6.98 (d, 1H, NH), 7.14-7.38 (m, 12H, 2 x Ph and NH_2), 7.68 (s, 1H, NH), 10.22 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{26}H_{34}N_7O_6$: 540.2571. Observed *m/z* 540.2575. Anal. Calcd. for $C_{26}H_{33}N_7O_6 \cdot 0.8H_2O$: C, 56.37; H, 6.29; N, 17.70. Found: C, 56.21; H, 6.29; N, 17.74.

(43, Mu-Ala-Ala-AAsn-COOCH$_2$Ph). Benzylchloroformate (1 eq) was added to a stirred solution of peptidyl hydrazide (1 eq) and pyridine (1.2 eq) in CH_2Cl_2 at -10 °C and the reaction mixture was stirred for 16 h at room temperature. The solvent was removed and the compound was purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (72% yield). ^{1}H NMR (DMSO-d_6): 1.10-1.17 (m, 6H, 2 x Ala-CH_3), 3.25 (m, 4H, morpholine), 3.34 (s, 2H, N*CH_2*CO), 3.50 (m, 4H, morpholine), 3.97 (m, 1H, α-H), 4.14 (m, 1H, α-H), 5.06 (s, 2H, *CH_2*Ph), 6.52 (d, 1H, NH), 7.19-7.32 (m, 6H, Ph and NH), 7.58 (s, 1H, NH), 7.95 (d, 1H, NH), 10.31 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{21}H_{31}N_6O_7$: 479.2254. Observed *m/z* 479.2264. Anal. Calcd. for $C_{21}H_{30}N_6O_7 \cdot 0.4H_2O$: C, 51.83; H, 6.40; N, 17.27. Found: C, 51.69; H, 6.34; N, 17.30.

CHAPTER 3

AZA-PEPTIDYL MICHAEL ACCEPTORS AND EPOXIDES AS INHIBITORS OF PARASITIC CYSTEINE PROTEASES

INTRODUCTION

Chagas' disease (American sleeping sickness) is the leading cause of heart disease in Latin American countries. The disease, transmitted by the protozoan parasite *Trypanosoma cruzi*, affects more than 25 million people annually in South America and causes more than 45,000 deaths per year.[71]

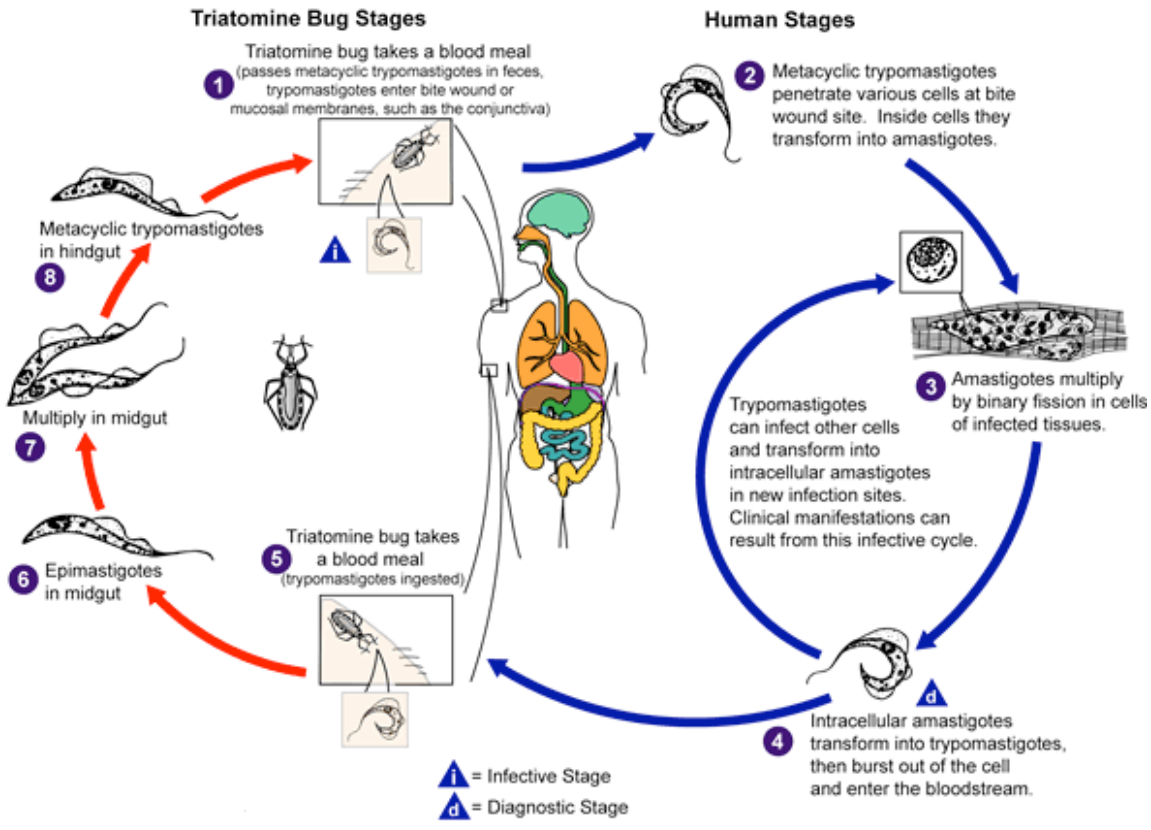


Figure 3.1. Life Cycle of *Trypanosoma cruzi*[72]

Trypanosoma cruzi is transmitted to humans when an infected insect vector takes a bite and releases trypomastigotes in its feces near the site of the bite wound. Trypomastigotes enter the host blood-stream through the wound or through intact mucosal membranes. Inside the host, the trypomastigotes invade cells near the site of inoculation and differentiate into intracellular amastigotes. The amastigotes multiply within cells, transform back to trypomastigotes, and then are released into the bloodstream. This cycle is repeated in each newly infected cell and clinical manifestations result from this infective cycle. Infection of the cardiac muscle cell leads to cardiomyopathy, while infection of the smooth muscle cells in the gut causes the destruction leading to enlarged or distended intestines.

Cruzain (also known as cruzipain) belongs to the family C1 of clan CA cysteine proteases. Cruzain was first purified in 1981[73] and has been shown to be expressed in the three developmental stages of *T. cruzi*.[74] Highest level of cruzain expression has been observed during the infective stage of the parasite.[75] Inhibition of cruzain has been shown to cure *T. cruzi* infection in cell culture screens and in mouse models of Chagas disease.[76] Several crystal structures of cruzain have been determined with both irreversible[14, 15] and reversible[16] inhibitors. Cruzain is the only parasite cysteine protease with a crystal structure. These crystal structures revealed the presence of the Glu205 residue located at the bottom of the S2 pocket. S2 pocket is the major specificity determinant for clan CA cysteine proteases and it has been shown that it can accommodate both hydrophobic residues like phenylalanine and basic residues like arginine. Presence of cruzain at all important stages of life cycle of the parasite makes it

an excellent target for the development of specific, irreversible inhibitors for the treatment of the disease.

African sleeping sickness or human African trypanosomiasis (HAT) is a major health problem in Equatorial Africa. It is estimated that more than 50,000 people are infected with the disease and 60 million people in 36 countries are at risk of infection; if left untreated, the disease is fatal in human.[77] *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense* are the two organisms responsible for the disease and they are transmitted by the bite of various species of the tsetse fly.

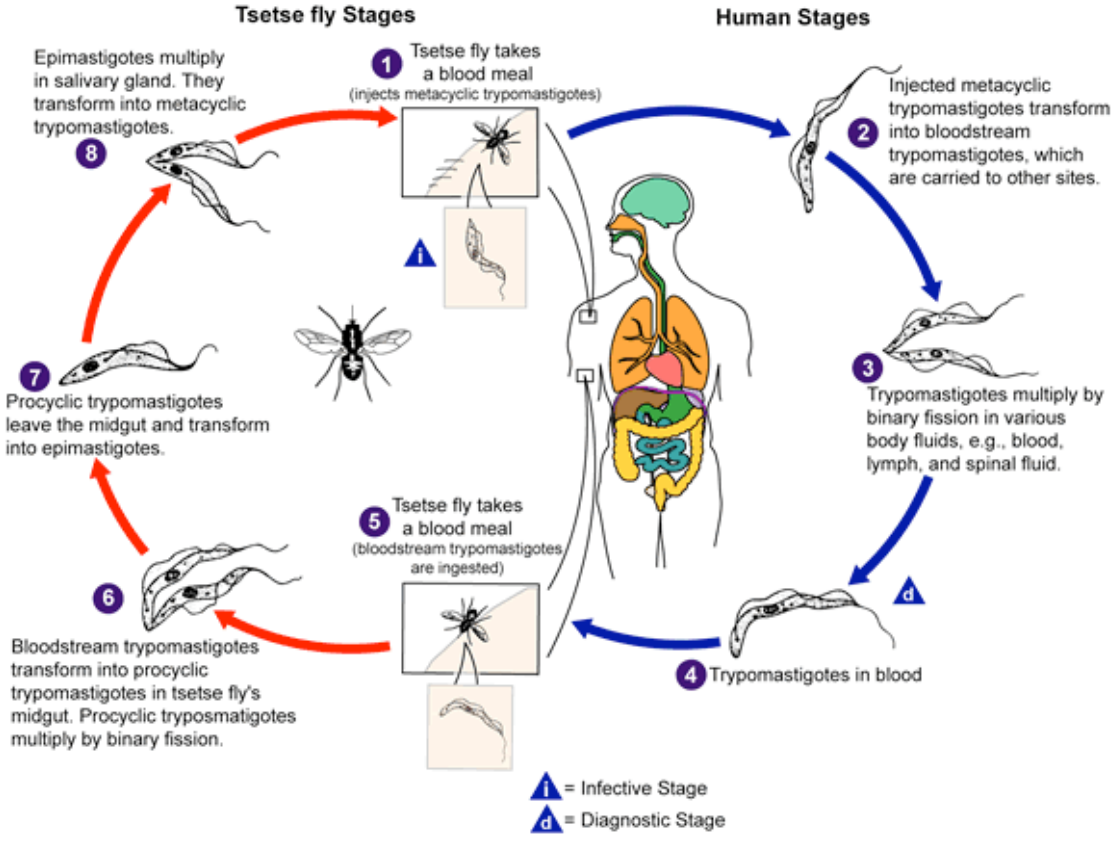


Figure 3.2. Life Cycle of *Trypanosoma brucei rhodesiense*[78]

When an infected tsetse fly takes a bite from the mammalian host, it injects metacyclic trypomastigotes into skin tissue. The parasites enter the lymphatic system and pass into the bloodstream. Once inside the host, they transform into bloodstream trypomastigotes and undergo more divisions. The entire life cycle of this parasite is represented by extracellular stages. The tsetse fly becomes infected by ingesting a blood meal from an infected mammalian host. The parasites transform into procyclic trypomastigotes in the fly's midgut, multiply, leave the midgut, and transform into epimastigotes. The epimastigotes reach the fly's salivary glands and continue to multiply. The cycle in the fly takes approximately 25-50 days and each fly remains infected for life.

Rhodesain is the major cysteine protease of *T. brucei rhodesiense* and has been identified in all life-cycle stages of the parasite,[79] it is also belong to clan CA of cysteine proteases and involves in regulating the replication of the parasite. Rhodesain is an important target for the development of antiparasitic chemotherapy since the inhibition of the enzyme will block the life cycle of the parasite in infected mammalian cells.

TbCatB is a recently discovered cathepsin B-like clan CA cysteine protease of *T. brucei*. It has been shown that TbCatB is required for host protein degradation in *T. brucei*[80] and therefore represents an exploitable target for the development of new inhibitors.

Current drug therapies for these diseases are either ineffective or have serious side effects, so there is an urgent need for the development of new medicinal agents. The synthetic inhibitors reported so far for cruzain, rhodesain and TbCatB include macrocyclic and peptidyl vinyl sulfones,[81-83] thiosemicarbazones,[84-86] peptidyl

aldehydes,[87] diazomethyl ketones,[88] fluoromethyl ketones,[76] peptidyl-α-β-epoxyesters,[89] aziridinyl peptides,[90] peptidyl allyl sulfones.[91]

INHIBITOR DESIGN

Our inhibitors were composed of two parts: The peptide sequence part that is involved in enzyme recognition and the warhead part which reacts with the active site cysteine of the cysteine proteases to inhibit the enzyme irreversibly; cruzain and rhodesain in this case. Aza-peptide epoxides[65, 92] and Michael acceptors[49, 93] have been shown to be potent inhibitors of clan CD cysteine proteases.

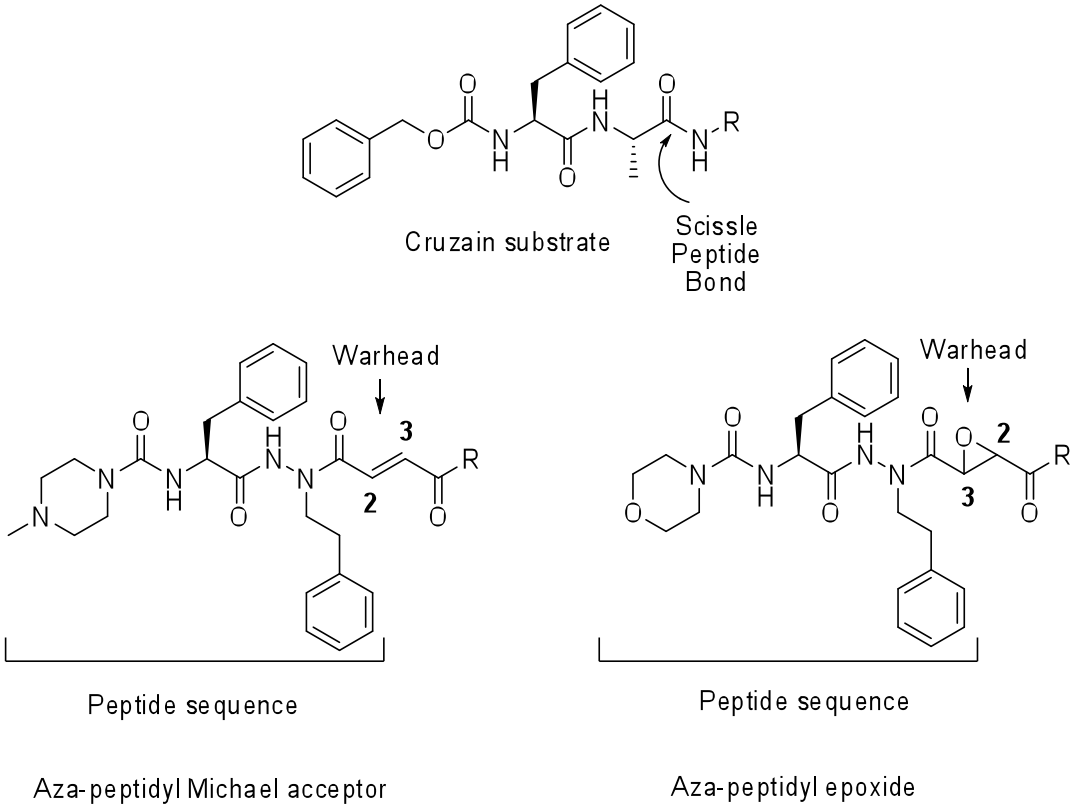


Figure 3.3. Design Strategy for Aza-peptide Michael Acceptor and Epoxide Inhibitors.

The inhibitors with epoxide and Michael acceptor warhead were designed to closely resemble an extended peptide substrate. Placement of the carbonyl group of the epoxide moiety or fumarate derivative in the inhibitor puts it in a location identical to that of the carbonyl of the scissile peptide bond in peptide substrate. This design allowed the peptide chain of the inhibitor to exactly match that of a good substrate up to the scissile peptide carbonyl group. Aza-peptide epoxides and Michael acceptors have the advantage of being easily extended in the P' direction, thus allowing interactions with the S' subsites of the enzyme.

There is no specific peptide sequence that these parasitic enzymes prefer, however they belong to Clan CA cysteine proteases and there are good inhibitors of Clan CA cysteine proteases with the peptide sequence Cbz-Leu-Abu.[94, 95] We decided to synthesize inhibitors with Cbz-Leu-AAbu peptide sequence where we change the α carbon of Abu to a nitrogen to form the aza-Abu (AAbu). Aza-peptides have the advantage of being easily extended in the P1' region by the attachment of Michael acceptor and epoxide warheads.

The second peptide sequence we focused on contained more hydrophobic amino acids; a phenylalanine (Phe) and an aza-homophenylalanine (AHph) in the P2 and P1 positions; respectively. Again we changed the α-carbon of homophenylalanine residue to nitrogen to form the aza-peptide. Aza-peptides are more resistant to enzymatic hydrolysis compared to amino acid analogs[81] and this replacement makes the synthesis of final compounds considerably easier since it involves the coupling of an acid and aza-peptide. There were inhibitors with Phe-Hph peptide sequence in the literature however they were using different warheads, like vinyl sulfones.[96] Currently, one of these

inhibitors, so called K11777 (Me-Piz-Phe-Hph-VS-Phenyl) has completed pre-clinical tests for the treatment of Chagas' disease and the inhibitor has been found to be non-toxic and non-mutagenic with an acceptable pharmacokinetic profile.[97] We have decided to incorporate *N*-methyl piperazine and morpholine into our peptide sequences as N-capping groups instead of Cbz group to both increase the bioavailability of our compounds and study the interactions in the P3 position.

Aza-peptide precursors (Cbz-Leu-NHNHCH$_2$CH$_3$, Mu-Phe-NHNHCH$_2$CH$_2$Ph and Me-Piz-Phe-NHNHCH$_2$CH$_2$Ph) were coupled to epoxides and Michael acceptors with a variety of substituents on the prime side such as esters, monosubstituted amides and disubstituted amides to study the effect of modification in the P1' position.

CHEMISTRY

For the synthesis of the aza-peptide precursor Cbz-Leu-NHNHCH$_2$CH$_3$, the methyl ester Cbz-Leu-OMe (**1**) was reacted with excess hydrazine (NH_2NH_2) in methanol to form the hydrazide Cbz-Leu-NH-NH$_2$ (**2**). The Abu side chain was introduced

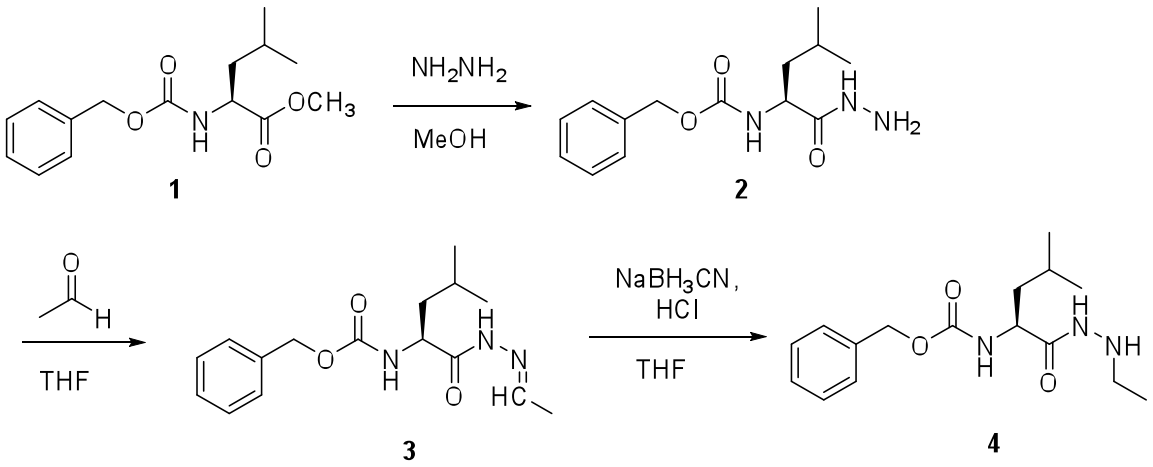


Figure 3.4. Synthesis of Cbz-Leu-NHNHCH$_2$CH$_3$.

reacting the hydrazide with acetaldehyde to form the hydrazone Cbz-Leu-NH-N=CH-CH_3 (**3**) which was reduced with $NaBH_3CN$[98] to form Cbz-Leu-$NHNHCH_2CH_3$ (Figure 3.4).

The synthesis of the acyl aza-homophenylalanine precursors (Y-CO-Phe-$NHNHCH_2CH_2Ph$) is shown in Figure 3.5. The hydrochloride salt of phenylalanine (**5**) was reacted with phosgene to form the phenylalanine ethyl ester isocyanate (**6**) using the procedure of Nowick et al.[99] The phenylalanine ethyl ester isocyanate (**6**) was then reacted with either morpholine or *N*-methyl piperazine to form Y-CO-Phe-OEt (**7, 8**). The ethyl ester Y-CO-Phe-OEt (**7, 8**) was then reacted with excess hydrazine (NH_2NH_2) in methanol to form the hydrazide Y-CO-Phe-NH-NH_2 (**9, 10**). Introduction of the aza-homophenylalanine side chain was achieved by first reacting the hydrazide Y-CO-Phe-NH-NH_2 with phenylacetaldehyde in THF to form the hydrazone (**11, 12**) and then reducing the hydrazone in the presence of $NaBH_3CN$ to yield the acyl aza-homophenylalanine precursors (**13, 14**).

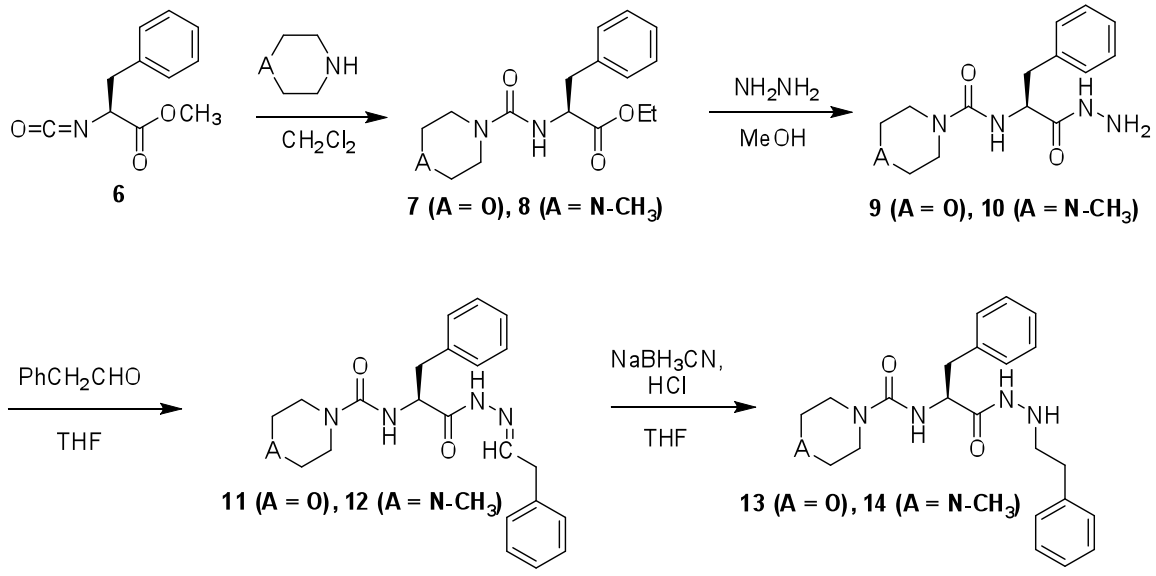
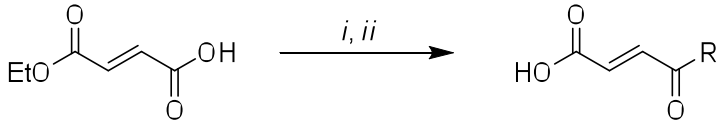


Figure 3.5. Synthesis of Acyl Aza-homophenylalanine Precursors.

The synthesis of the aza-peptide fumarate and epoxide analogs are based on the previous syntheses of aza-peptide Michael acceptor[93] and epoxide[92] inhibitors. Monoethyl fumarate (**15**) was commercially available and all the Michael acceptor warheads were synthesized using this compound as a precursor. The fumarate precursors (**16 a-k**) were prepared from monoethyl fumarate and the corresponding primary or secondary amines by standard mixed anhydride coupling procedure using NMM and iBCF; and followed by hydrolysis of the ethyl ester with aqueous NaOH (Figure 3.6).



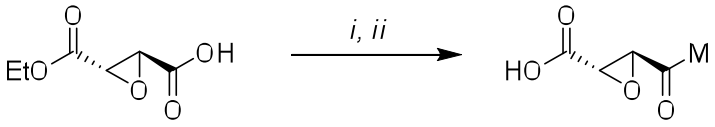
15 **16 a-k**

R =

a = -OEt
b = -N(CH$_3$)Ph
c = -NHCH$_2$Ph
d = -NHCH$_2$Ph-4-F
e = -NH(CH$_2$)$_2$Ph
f = -NH(CH$_2$)$_3$Ph
g = -NHCH$_2$-1-naphthyl
h = -N(CH$_3$)CH$_2$-1-naphthyl
i = -NH(CH$_2$)$_2$indole
j = -N(CH$_2$Ph)$_2$
k= -NH(CH$_2$)$_2$CH(Ph)$_2$

Reagents: (*i*) NMM, iBCF, CH_2Cl_2, HNR_1R_2; (*ii*) 1. NaOH, 2. HCl, EtOH.

Figure 3.6. Synthesis of Fumarate Precursors.



17 **18l, m**

M =

l = -NHCH$_2$-1-naphthyl
m = -N(CH$_2$Ph)$_2$

Reagents: (*i*) NMM, iBCF, CH_2Cl_2, HNR_1R_2; (*ii*) 1. KOH, 2. HCl, EtOH.

Figure 3.7. Synthesis of Epoxysuccinate Derivatives.

A previously described procedure[92] was followed for the synthesis of (*2S,3S*)-oxirane-2,3-dicarboxylic acid monoethyl ester (**17**). The epoxide warheads (**18l, m**) were synthesized from the monoethyl ester epoxysuccinate and the corresponding primary or secondary amines by using the standard mixed anhydride coupling procedure using NMM and iBCF followed by hydrolysis of the ethyl ester in ethanol using aqueous KOH (Figure 3.7.).

The aza-peptidyl inhibitors were obtained by coupling aza-peptidyl precursor to a fumarate acid or an epoxysuccinyl acid derivative using HOBt and EDC (Figure 3.8).

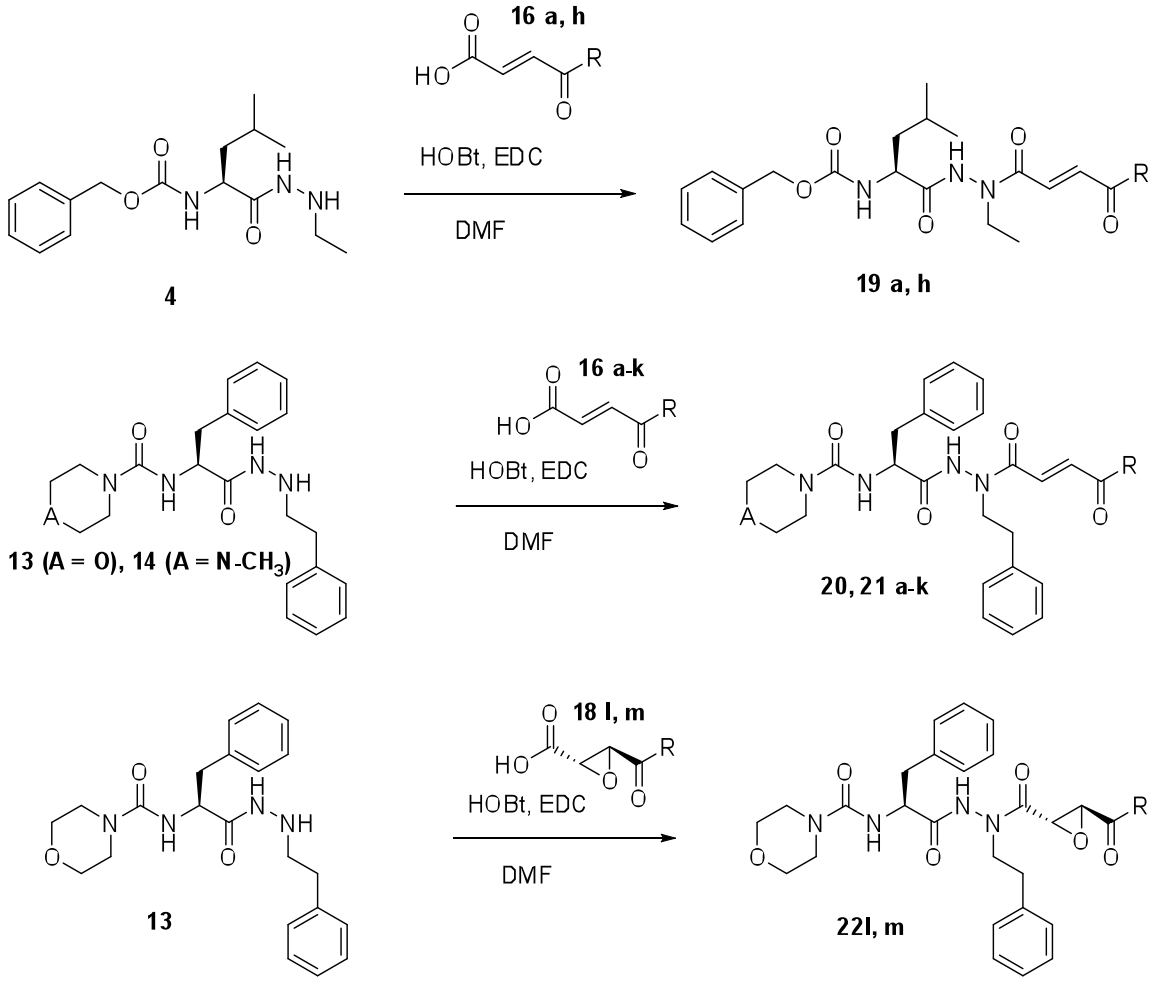


Figure 3.8. Coupling of Fumarate Precursors and Epoxide Moieties to Aza-peptide Precursors.

RESULTS AND DISCUSSION

Inhibition Studies

We synthesized two Michael acceptor inhibitors with Cbz-Leu-AAbu peptide sequences and the ethyl ester derivative (**19a**) with an IC_{50} of 14 μM against cruzain has the same potency as the disubstituted amide derivative (**19h**) with an IC_{50} of 16 μM. Rhodesain favored the ethyl ester derivative with an IC_{50} of 4 μM to the disubstituted amide derivative with an IC_{50} of 20 μM. Both compounds were moderate inhibitors of cruzain and rhodesain and inactive against TbCatB but displayed some trypanocidal activity with an IC_{50} of 55 μM for compound **19a** and 18 μM for compound **19h**.

From the five inhibitors with Mu-Phe-AHph peptide sequence, three of them were Michael acceptor inhibitors and two of them were epoxide inhibitors. Among the three Michael acceptor inhibitors, the one with disubstituted amide derivative (**20b**, IC_{50} >100 μM) was a poor inhibitor of both cruzain and rhodesain. When the methyl group was replaced with hydrogen in compound **20c**, IC_{50} value of 0.9 μM against cruzain and IC_{50} value of 2 μM against rhodesain was obtained. This result indicated the importance of the amide hydrogen in that position and a possible involvement in hydrogen bonding with an amino acid residue of the active site of both enzymes. Mu-Phe-AHph-CH=CH-$CONH(CH_2)_2CH(Ph)_2$ was a poor inhibitor of cruzain and rhodesain (IC_{50} >100 μM) even though it was a monosubstituted amide analog. We speculated that the alkyl spacer and the two phenyl rings may position the inhibitor away from the hydrogen bond

Table 3.1. Inhibition of Cruzain, Rhodesain, TbCatB and *T. brucei* by Aza-Peptide Michael Acceptors and Epoxides

Compound	Cruzain IC_{50} (μM)	Rhodesain IC_{50} (μM)	*T. brucei* IC_{50} (μM)
Cbz-Leu-AAbu-CH=CH-COOEt	14	4	55
Cbz-Leu-AAbu-CH=CH-CON(CH_3)CH_2-1-naphthyl	16	20	18
Mu-Phe-AHph-CH=CH-CON(CH_3)Ph	>100	>100	23
Mu-Phe-AHph-CH=CH-CONHCH_2Ph	0.9	2	18
Mu-Phe-AHph-CH=CH-CONH$(CH_2)_2$CH$(Ph)_2$	>100	>100	11.5
Mu-Phe-AHph-EP(*S,S*)-CONHCH_2-1-naphthyl	0.09	0.04	7
Mu-Phe-AHph-EP(*S,S*)-CON$(CH_2Ph)_2$	3	1	6
Me-Piz-Phe-AHph-CH=CH-CON(CH_3)Ph	>100	>100	77
Me-Piz-Phe-AHph-CH=CH-CONHCH_2Ph	4	0.8	29
Me-Piz-Phe-AHph-CH=CH-CONHCH_2Ph-4-F	0.2	0.02	26
Me-Piz-Phe-AHph-CH=CH-CONH$(CH_2)_2$Ph	2.8	0.16	17
Me-Piz-Phe-AHph-CH=CH-CONH$(CH_2)_3$Ph	37	0.08	16.2
Me-Piz-Phe-AHph-CH=CH-CONHCH_2-1-naphthyl	3	0.5	5.6
Me-Piz-Phe-AHph-CH=CH-CON(CH_3)CH_2-1-naphthyl	>100	>100	5.6
Me-Piz-Phe-AHph-CH=CH-CONH$(CH_2)_2$indole	98	1.2	22
Me-Piz-Phe-AHph-CH=CH-CON$(CH_2Ph)_2$	>100	>100	6.6
Me-Piz-Phe-AHph-CH=CH-CONH$(CH_2)_2$CH$(Ph)_2$	52	87	11

network and two phenyl rings may not be accommodated in the P1' position due to bulkiness of the groups. Mu-Phe-AHph-EP(*S,S*)-$CONHCH_2$-1-naphthyl was one of the two epoxide inhibitors synthesized and yielded the most potent inhibitor in this series with an IC_{50} of 0.09 μM against cruzain and IC_{50} of 0.04 μM against rhodesain. The second epoxide inhibitor Mu-Phe-AHph-EP(*S,S*)-$CON(CH_2Ph)_2$, was a disubstituted amide derivative and resulted in decrease in potency with an IC_{50} = 3 μM against cruzain and IC_{50} of 1 μM against rhodesain. All the compounds with Mu-Phe-AHph peptide sequence have IC_{50} values higher than 100 μM against TbCatB. In the proliferation assay, aza-peptide epoxides, which had large and bulky hydrophobic groups in the P1' position, were favored over aza-peptide Michael acceptors.

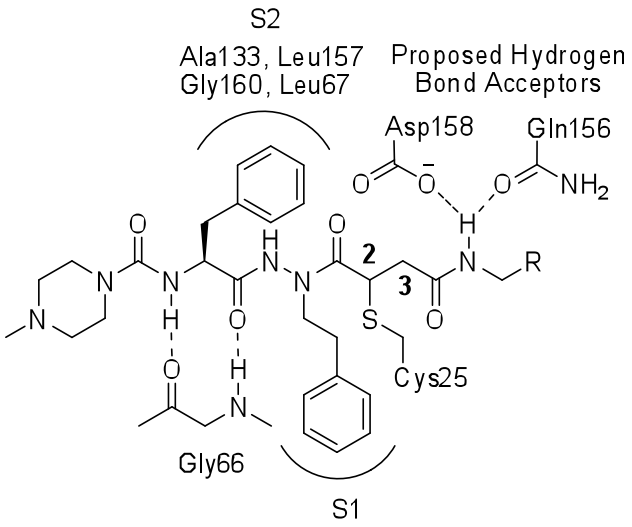


Figure 3.9. Proposed Mode of Interaction of the Aza-peptide Michael Acceptor Inhibitor with the Active Site of Cruzain

We have synthesized 10 Michael acceptor inhibitors with Me-Piz-Phe-AHph peptide sequence. Three compounds (**21b, 21, h, 21j**) with disubstituted amide analogs

were poor inhibitors of both cruzain and rhodesain with IC_{50} values greater than 100 μM. Replacement of the methyl group in compound **21h** with hydrogen to obtain compound **21g** (Me-Piz-Phe-AHph-CH=CH-CONHCH$_2$-1-naphthyl, IC_{50} = 3 μM against cruzain and IC_{50} = 0.5 μM against rhodesain) resulted in increased potency again suggesting the importance of amide hydrogen in this position. Indeed, analysis of the crystal structures from the vinyl sulfone inhibitors showed that the side chains of Asp158 and Gln156 were positioned toward the amide NH. Based on the kinetic data and the crystal structures available, we proposed that the amide nitrogen is forming hydrogen bonds with Asp158 and Gln156 side chains (Figure 3.9). Me-Piz-Phe-AHph-CH=CH-CONH$(CH_2)_2$CH$(Ph)_2$ have IC_{50} values of 52 and 87 μM against cruzain and rhodesain; respectively. This compound was slightly more potent than the disubstituted amide analogs but still weak inhibitor of cruzain and rhodesain due to the presence of bulky hydrophobic groups. Me-Piz-Phe-AHph-CH=CH-CONH$(CH_2)_2$indole was a weak inhibitor of cruzain with an IC_{50} of 98 μM whereas it was a good inhibitor of rhodesain with an IC_{50} of 1.2 μM. This is one of the two examples where the two parasite enzymes differed markedly in their SAR. There is an NH in the indole ring of this compound (**21i**) and the hydrogen is probably involving in a hydrogen bonding network near the active site of rhodesain. Me-Piz-Phe-AHph-CH=CH-CONH$(CH_2)_3$Ph (**21f**) was also a weak inhibitor of cruzain with an IC_{50} value of 37 μM, probably due to the unfavorable positioning of the phenyl ring by the alkyl spacer. However, in the case of rhodesain, **21f** was one of the most potent compounds synthesized in this series against this enzyme with an IC_{50} of 0.08 μM. As the length of the alkyl spacer decreased in compounds **21e** (Me-Piz-Phe-AHph-CH=CH-CONH$(CH_2)_2$Ph, IC_{50} = 2.8 μM and **21c** (Me-Piz-Phe-AHph-CH=CH-CONHCH$_2$Ph,

IC_{50} = 4 μM) the inhibition potency of the compounds against cruzain increased. On the other hand, shortening of the alkyl chain by one methylene group resulted in 2-fold decrease in potency in compound **21e** (IC_{50} = 0.16 μM) and further shortening of the alkyl spacer by removal of another methylene group resulted in 5-fold decrease in potency in compound **21c** (IC_{50} = 0.8 μM). We hypothesized that the length of the alkyl spacer was important in positioning the aromatic phenyl group and that phenyl group is reaching a hydrophobic pocket in the S2'. Introduction of an electron withdrawing fluorine into the phenyl ring led to the discovery of most potent Michael acceptor inhibitor for both cruzain and rhodesain with IC_{50} values of 0.2 μM and 0.02 μM; respectively. All the compounds with Me-Piz-Phe-AHph peptide sequence have IC_{50} values greater than 100 μM against TbCatB. On the other hand, all the compounds inhibited parasite proliferation. Most potent compounds displayed trypanocidal activity in the low micromolar range and it has been observed that as we increased the hydrophobicity in the P1' position, the activity against the parasite increased. In a recent study with purine-derived nitrile inhibitors, it has been observed that the trypanocidal activity against the parasite increased with increasing the hydrophobicity of the compounds.[100] Two compounds were in particular interest. Compounds **21h** and **21j** had IC_{50} values greater than 100 μM against all the enzymes, but inhibited the parasite proliferation with IC_{50} values of 5.6 μM and 6.6 μM; respectively.

Mechanism of Inhibition. Aza-peptide Michael acceptors and epoxides are irreversible inhibitors of clan CD cysteine proteases. The mechanism of inhibition of cysteine proteases by Michael acceptors and epoxides proceeds via a nucleophilic attack

of the catalytic cysteine residue on the double bond of the Michael acceptor or the epoxide ring, followed by the formation of a covalent bond, and the inhibition of the enzyme irreversibly. With the present inhibitors, nucleophilic attack of the active site cysteine thiol could occur at either carbon (C2 or C3) of the Michael acceptor double bond (Figure 3.10). Crystal structures of aza-peptide epoxide inhibitors specifically designed to inhibit caspase-3 showed that the cysteine thiol attack occurs at the C3 carbon of the epoxide.[65] Crystal structures of several Michael acceptor inhibitors of caspase-3 and caspase-8 have been determined.[64] It has been observed that the cysteine thiol attack occurs at the C2 carbon of the Michael acceptor double bond. As a result, we conclude that it is likely that cruzain and rhodesain are also alkylated at the C2 carbon of Michael acceptor double bond and C3 carbon of epoxide inhibitors. Thus, the site of alkylation is located at approximately the same distance from the aza-peptide nitrogen atom in both aza-peptide epoxides and aza-peptide Michael acceptor inhibitors.

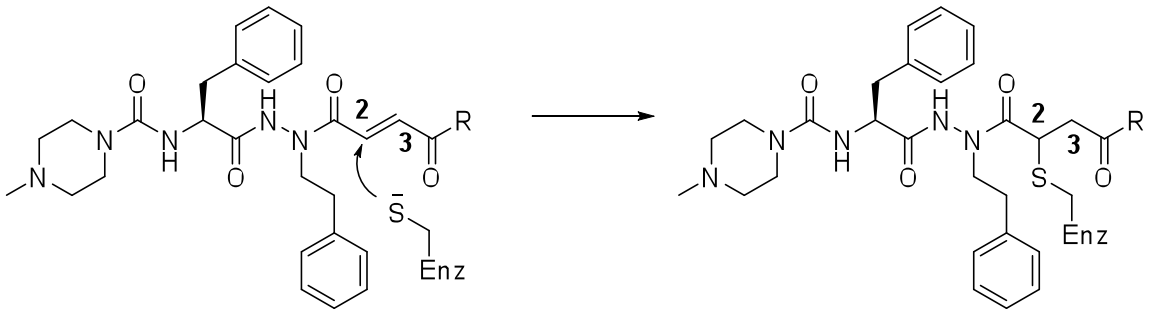


Figure 3.10. Proposed Mechanism of Inhibition of Cruzain by Aza-peptidyl Michael Acceptor

CONCLUSION

We have demonstrated the potential of aza-peptidyl Michael acceptors and epoxides to inhibit parasitic cysteine proteases; cruzain and rhodesain and also inhibit the parasite proliferation. We have shown that compounds with Mu-Phe-AHph peptide sequence are favored over Me-Piz-Phe-AHph and that, in turn, are favored over Cbz-Leu-AAbu peptide sequence.

Aza-peptide epoxides were potent inhibitors with IC_{50} values as low as 90 nM and 40 nM against cruzain and rhodesain; respectively. With aza-peptide Michael acceptors, we have also obtained IC_{50} values in the nanomolar range. We have observed that monosubstituted amides were preferred to disubstituted amides indicating the importance of hydrogen bonding in that position. Hence, the study of the available crystal structures of cruzain showed two possible hydrogen bonding amino acid residues, Asp158 and Gln156, near the active site. It has been shown before that the phenylalanine residue in the P2 position is interacting with the hydrophobic residues Leu67, Ala133, Leu157 and Gly160 and hydrogen bonds are formed between the inhibitor's peptide backbone atoms (amide nitrogen and carbonyl oxygen of the P2 residue) and Gly66 in the catalytic cleft. Here we report for the first time the involvement of the amide hydrogen in the P1' position in a hydrogen network. We have observed that as we increased the length of the alkyl spacer of the hydrophobic groups, the potency decreased in cruzain but it had little effect in rhodesain.

Irreversible cysteine protease inhibitors would have great potential for the short-term therapeutic administration against parasitic infections. Recently, a Michael acceptor

inhibitor, K11777 (Me-Piz-Phe-Hph-VS-Phenyl) has completed pre-clinical trials for the treatment of Chagas' disease. Here, we have shown that aza-peptide epoxide and Michael acceptor inhibitors represent promising drug leads capable of inhibiting the parasitic enzymes in the nanomolar range. Also, there has been no success in determination of a crystal structure of rhodesain, and selective irreversible inhibitors reported here may help to determine the structural and enzymatic specificities of the rhodesain active site.

Significance

African sleeping sickness and Chagas' disease affect millions of people in South America and Africa. Currently used drugs have serious side effects and the parasite is becoming more and more resistant to these drugs, and therefore there is an urgent need for the development of new anti-parasitic agents. This research expanded our knowledge about the active sites of the parasitic cysteine proteases, rhodesain and cruzain. We have discovered new sites of interaction of inhibitors with these parasitic cysteine proteases. This research will assist in the development of potent inhibitors which in turn will help millions of sick people who are infected with these parasites.

EXPERIMENTAL

Material and Methods. Materials were obtained from Acros, Bachem Bioscience Inc., or Sigma Aldrich and used without further purification. The purity of each compound was confirmed by TLC, ^{1}H NMR, MS, and elemental analysis. Chemical shifts are reported in ppm relative to an internal standard (trimethylsilane). TLC was performed on Sorbent Technologies (250 μm) silica gel plates. The ^{1}H NMR

spectra were obtained on a Varian Mercury 400 MHz spectrometer. Electrospray ionization (ESI), fast-atom-bombardment (FAB) and high-resolution mass spectrometry were obtained using Micromass Quattro LC and VG Analytical 70-SE instruments. Elemental analysis was carried out by Atlantic Microlab Inc., Norcross, GA. The synthetic aza-peptide inhibitors were tested against cruzain, rhodesain, TbCatB and *T. brucei* by Conor Caffrey in the laboratory of Jim McKerrow at the University of California, San Francisco.

Cruzain. Inhibitors were screened for effectiveness against the *T. cruzi* cathepsin L-like protease (cruzain) using purified recombinant protein. Cruzain (1 nM) was incubated with 20-50000 nM inhibitor in 100 mM sodium acetate, 5 mM DTT buffer (pH 5.5) for 5 min at room temperature. An aliquot of Cbz-Phe-Arg-AMC (200 μL) was added to the enzyme-inhibitor reaction to give a 20 μM substrate concentration. The increase in fluorescence (excitation at 355 nM and emission at 460 nM) was followed with an automated microtiter plate spectrofluorimeter (Molecular Devices, SpectraMAX Gemini). Inhibitor stock solutions were prepared at 20 mM in DMSO, and serial dilutions were made in DMSO. Controls were performed using enzyme alone and enzyme with DMSO.

Rhodesain. Rhodesain (3 nM) was incubated with 0.5 to 10 μM inhibitor in 100 mM sodium acetate, containing 5 mM DTT, pH 5.5 (Buffer A) for 5 min at room temperature. Then buffer A containing Z-Phe-Arg-AMC was added to enzyme inhibitor to give 20 μM substrate in 200 μL, and the increase in fluorescence (excitation at 355 nM and emission at 460 nM) was followed with an automated microtiter plate spectrofluorimeter (Molecular Devices, Flex station). Inhibitor stock solutions were

prepared at 20 mM in DMSO, and serial dilutions were made in DMSO. Controls were performed using enzyme alone and enzyme with DMSO.

TbcatB. An amount of 100 μL of TbcatB (final assay concentration, 35 nM) in assay buffer (0.05 M sodium acetate, pH 5.5, 4 mM DTT, 0.5 mM EDTA) was added to each well of a black flatbottom polystyrene 96-well plate (Corning). An amount of 2 μL of inhibitor in DMSO was added, and the mixture was incubated at room temperature for 20 min before the addition of 100 μL of Z-Phe-Arg-AMC (final assay concentration, 10 μM) substrate in assay buffer. Initial rates were determined by monitoring fluorescence (λ_{ex} = 350 nm, λ_{em} = 460 nm) on an Envision plate reader (Perkin-Elmer) for 10 min.

Cell Proliferation Assays. All IC_{50} values were determined by curve-fitting with GraphPad Prism software. Culture-adapted *T. brucei* were grown at 37 °C in 5% CO_2 in HMI-9 medium (HyClone) supplemented with penicillin/streptomycin (50 units/mL), 10% heat-inactivated FBS (Omega Scientific, lot no. 3137), and 10% Serum Plus (JHR Biosciences) to a density of 1 × 106 cells/mL and then diluted to 1 × 104 cells/mL. An amount of 100 μL of the diluted culture was added to each well of a white flat-bottom sterile 96-well plate (Greiner), and 1 μL of inhibitor in DMSO was added. Plates were incubated for 48 h at 37 °C, in 5% CO_2, and then equilibrated at room temperature for 1 h before the addition of 100 μL of Cell Titer Glo (Promega) to each well. Plates were then shaken on an orbital shaker for 2 min at 500 rpm. Luminescence was read after 8 min on an Envision plate reader (Perkin-Elmer).

General Procedure for the Synthesis of Acyl Phenylalanine Ethyl ester. Phenylalanine methyl ester isocyanate, which was synthesized from the hydrochloride salt of phenylalanine according to the procedure by Nowick et al., was dissolved in

CH_2Cl_2 and cooled down to 0 °C. Morpholine or *N*-methylpiperazine was added to the stirred solution of phenylalanine ethyl ester isocyanate in dichloromethane. Reaction mixture was stirred vigorously for 16 h at room temperature. Solvent was removed under vacuum and the crude product was purified by column chromatography.

(S)-ethyl 2-(morpholine-4-carboxamido)-3-phenylpropanoate (7, Mu-Phe-OEt) was purified by column chromatography on silica gel using 4:1 EtOAC:Hexane as the eluent; white solid, yield 63%. ^{1}H-NMR ($CDCl_3$-d): 1.21-1.25 (t, 3H, CH_3), 3.10 (d, 2H, CH_2Ph), 3.27-3.32 (m, 4H, morpholine), 3.62-3.65 (m, 4H, morpholine), 4.13-4.18 (m, 2H, CH_2), 4.74-4.78 (m, 1H, α-H), 4.91 (d, 1H, NH), 7.10-7.29 (m, 5H, Ph). MS (ESI) *m/z* 307.0 [$(M + 1)^+$].

(S)-ethyl 2-(4-methylpiperazine-1-carboxamido)-3-phenylpropanoate (8, Me-Piz-Phe-OEt) was purified by column chromatography on silica gel using 10%MeOH/CH_2Cl_2 as the eluent; white solid, yield 81%. ^{1}H-NMR ($CDCl_3$-d): 1.20-1.23 (t, 3H, CH_3), 2.27-2.35 (m, 7H, piperazine and NCH_3), 3.09 (d, 2H, CH_2Ph), 3.29-3.38 (m, 4H, piperazine), 4.11-4.17 (m, 2H, CH_2), 4.72-4.89 (m, 1H, α-H), 4.90 (d, 1H, NH), 7.09-7.28 (m, 5H, Ph). MS (ESI) *m/z* 320.1 [$(M + 1)^+$].

Peptidyl Hydrazides. Anhydrous hydrazine (10 equiv) was added to a stirred solution of a peptidyl ethyl ester (1 eq) or N-benzyloxycarbonyl leucine methyl ester (Cbz-Leu-OMe) in MeOH and the reaction mixture was stirred vigorously for 16 h at room temperature. Excess hydrazine and solvent were removed under vacuum and the residue was washed with ether several times to give the peptidyl hydrazide as a white solid.

Cbz-Leu-NH-NH_2 (**2**), white solid, 64% yield. ^{1}H NMR (DMSO-d_6): 0.81-0.88 (m, 6H, 2 x Leu-CH_3), 1.29-1.58 (m, 3H, CH_2 and CH), 3.96 (m, 1H, α-H), 4.19 (s, 2H, NH_2), 4.47 (d, 1H, NH), 4.99 (s, 2H, CH_2), 7.29-7.39 (m, 5H, Ph), 9.15 (s, 1H, NH). MS (ESI) *m/z* 280.2 [$(M + 1)^+$].

Mu-Phe-NH-NH_2 (**9**), white solid, 93% yield. ^{1}H-NMR (DMSO-d_6): 2.78-2.91 (m, 2H, CH_2Ph), 3.12-3.25 (m, 4H, morpholine), 3.39-3.49 (m, 4H, morpholine), 4.19-4.29 (m, 3H, α-H and NH_2), 6.57 (d, 1H, NH), 7.14-7.24 (m, 5H, Ph), 9.10 (s, 1H, NH). MS (ESI) *m/z* 293.1 [$(M + 1)^+$].

Me-Piz-Phe-NH-NH_2 (**10**), white solid, 95% yield. ^{1}H-NMR (DMSO-d_6): 2.11-2.48 (m, 7H, piperazine and NCH_3), 2.86-2.91 (m, 2H, CH_2Ph), 3.20-3.23 (m, 4H, piperazine), 3.55 (s, 2H, NH_2), 4.23 (m, 1H, α-H), 6.52 (d, 1H, NH), 7.15-7.34 (m, 5H, Ph), 10.97 (s, 1H, NH). MS (ESI) *m/z* 306.1 [$(M + 1)^+$].

Peptidyl Hydrazones. Phenylacetaldehyde or acetaldehyde (2 eq) was added to a stirred solution of peptidyl hydrazide (1 eq) in dry THF and the reaction mixture was stirred overnight at room temperature. Solvent was removed under vacuum and the crude product was purified by column chromatography.

Cbz-Leu-NHN=$CHCH_3$ (**3**) was purified by column chromatography on silica gel using 10%MeOH/CH_2Cl_2 as the eluent; white solid, yield 92%. ^{1}H NMR (DMSO-d_6): 0.82-0.88 (m, 9H, 2 x Leu-CH_3 and Abu-CH_3), 1.32-1.64 (m, 3H, CH_2 and CH), 3.95-3.99 (m, 1H, α-H), 4.99 (s, 2H, CH_2), 7.26-7.33 (m, 6H, Ph and CH), 7.49 (d, 1H, NH), 10.85 (s, 1H, NH). MS (ESI) *m/z* 306.1 [$(M + 1)^+$].

Mu-Phe-NHN=$CHCH_2$Ph (**11**) was purified by column chromatography on silica gel using 10%MeOH/CH_2Cl_2 as the eluent; yellow solid, yield 73%. ^{1}H-NMR (DMSO-

d_6): 3.14-3.47 (m, 10H, morpholine and CH_2Ph), 3.58 (d, 2H, CH*CH_2*Ph), 4.21-4.26 (m, 1H, α-H), 6.59 (d, 1H, NH), 7.15-7.34 (m, 10H, 2 x Ph), 7.55 (t, 1H, CH), 10.99 (s, 1H, NH). MS (ESI) *m/z* 395.2 [$(M + 1)^+$].

Me-Piz-Phe-NHN=$CHCH_2$Ph (**12**) was purified by column chromatography on silica gel using 15%MeOH/CH_2Cl_2 as the eluent; yellow solid, yield 75%. ^{1}H-NMR (DMSO-d_6): 2.18-2.30 (m, 7H, piperazine and NCH_3), 3.01-3.44 (m, 6H, piperazine and CH*CH_2*Ph), 3.52-3.61 (m, 2H, CH_2Ph), 4.54-4.60 (m, 1H, α-H), 5.55 (d, 1H, NH), 7.11-7.31 (m, 11H, 2 x Ph and CH), 10.51 (s, 1H, NH). MS (ESI) *m/z* 408.2 [$(M + 1)^+$].

Aza-peptidyl precursor. $NaBH_3CN$ (3 eq) was added to a solution of peptidyl hydrazone (1 eq) in THF. The reaction mixture was heated to 60 °C and stirred overnight after the addition of HCl. The solvent was evaporated and the residue was dissolved in EtOAc and washed with 10% $NaHCO_3$, water, saturated NaCl and dried over $MgSO_4$ and concentrated. Purification on a silica gel column with the proper eluent gave the product with yields of 52-72%.

Cbz-Leu-$NHNHCH_2CH_3$ (**4**) was purified by column chromatography on silica gel using 10%MeOH/CH_2Cl_2 as the eluent; white solid, yield 52%. ^{1}H NMR (DMSO-d_6): 0.81-0.94 (m, 9H, 2 x Leu-CH_3 and Abu-CH_3), 1.33-1.57 (m, 3H, CH_2 and CH), 2.62-2.66 (m, 2H, CH_2), 3.97 (m, 1H, α-H), 4.76 (s, 1H, NH), 4.99 (s, 2H, CH_2), 7.30-7.39 (m, 6H, Ph and NH), 9.41 (s, 1H, NH). MS (ESI) *m/z* 308.2 [$(M + 1)^+$].

Mu-Phe-$NHNHCH_2CH_2$Ph (**13**) was purified by column chromatography on silica gel using 10%MeOH/CH_2Cl_2 as the eluent; white solid, yield 68%. ^{1}H-NMR (DMSO-d_6): 2.60 (t, 1H, NH), 2.81-2.88 (m, 4H, *CH_2*CH_2Ph and CH_2*CH_2*Ph), 3.15-3.23 (m, 6H,

morpholine and *CH*$_2$Ph), 3.41-3.49 (m, 4H, morpholine), 4.23-4.28 (m, 1H, α-H), 6.61 (d, 1H, NH), 7.13-7.29 (m, 10H, 2 x Ph), 9.43 (s, 1H, NH). MS (ESI) *m/z* 397.2 [(M + 1)$^+$].

Me-Piz-Phe-NHNHCH$_2$CH$_2$Ph (**14**) was purified by column chromatography on silica gel using 15%MeOH/CH_2Cl_2 as the eluent; white solid, yield 72%. ^{1}H-NMR (DMSO-d$_6$): 2.25-2.31 (m, 7H, piperazine and NCH$_3$), 2.61-2.64 (t, 2H, NH*CH*$_2$CH$_2$Ph), 2.87-3.05 (m, 4H, *CH*$_2$Ph and CH$_2$*CH*$_2$Ph), 3.27-3.32 (m, 4H, piperazine), 4.47-4.55 (m, 1H, α-H), 5.24 (d, 1H, NH), 7.12-7.32 (m, 11H, 2 x Ph and NH), 8.19 (s, 1H, NH). MS (ESI) *m/z* 410.3 [(M + 1)$^+$].

General Procedure for the Synthesis of Fumaric Acid Monoamides by the Mixed Anhydride Coupling Method. Coupling of the amine precursors to monoethyl fumarate was accomplished using the mixed anhydride coupling method. To a solution of the monoethyl fumarate (1 eq) in CH_2Cl_2 at -20 °C was added N-methylmorpholine (NMM, 1 eq) followed by isobutyl chloroformate (iBCF, 1 eq). After the reaction mixture was allowed to stir for 30 min, the amine (1 eq) was added to the mixture. Hydrochloride salts of the amine were pretreated with NMM (1 eq) at -20 °C in CH_2Cl_2 prior to addition. After 30 min the reaction was continued to stir overnight at room temperature. The methylene chloride was evaporated and the residue was redissolved in ethyl acetate and washed with 2% citric acid, saturated $NaHCO_3$, saturated NaCl, dried over $MgSO_4$, and concentrated. The product was purified by column chromatography as needed.

***trans*-N-Methyl-N-phenylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH=CHCON(CH$_3$)Ph)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and N-methyl aniline to give a white solid

(99% yield). ^{1}H-NMR ($CDCl_3$): 1.25 (t, 3H, *CH_3*CH_2), 3.39 (s, 3H, N-CH_3), 4.17 (q, 2H, CH_3*CH_2*), 6.93-6.97 (d, 1H, *J* = 15.2 Hz, *CH*=CHCON), 7.10-7.14 (d, 1H, *J* = 15.2 Hz, CH=*CH*CON), 7.15 (t, 1H, Ph), 7.34 (t, 2H, Ph), 7.61 (d, 2H, Ph).

***trans*-N-Methyl-N-phenylcarbamoylpropenoic Acid (16b, HOOCCH=CHCON(CH_3)Ph).** EtOOCCH=CHCON(CH_3)Ph was hydrolyzed in MeOH using NaOH (1 M aqueous, 1.1 eq) under standard deblocking conditions to give a white solid (29% yield). ^{1}H-NMR (DMSO-d_6): 3.14 (s, 3H, N-CH_3), 6.50-6.54 (d, 1H, *J* = 15.2 Hz, *CH*=CHCON), 6.60-6.64 (d, 1H, *J* = 15.2 Hz, CH=*CH*CON), 7.32 (t, 2H, Ph), 7.40 (d, 1H, Ph), 7.47 (d, 1H, Ph).

***trans*-3-Benzylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH=CHCONHCH$_2$Ph)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and benzylamine. Purification by column chromatography using 5% MeOH/CH_2Cl_2 gave a white powder (81% yield). ^{1}H-NMR (DMSO-d_6): 1.20-1.24 (t, 3H, CH_2*CH_3*), 4.14-4.19 (q, 2H, *CH_2*CH_3), 4.37 (d, 2H, N*CH_2*Ph), 6.57-6.61 (d, 1H, *CH*=CHCON), 7.03-7.07 (d, 1H, CH=*CH*CON), 7.22-7.34 (m, 5H, Ph), 9.04 (t, 1H, NH).

***trans*-3-Benzylcarbamoylpropenoic Acid (16c, HOOCCH=CHCONHCH$_2$Ph).** EtOOCCH=CHCONHCH$_2$Ph was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 eq) under standard deblocking conditions to give a white solid (73% yield). ^{1}H-NMR (DMSO-d_6): 4.36 (d, 2H, N*CH_2*Ph), 6.52-6.56 (d, 1H, *CH*=CHCON), 6.95-6.99 (d, 1H, CH=*CH*CON), 7.23-7.33 (m, 5H, Ph), 8.98 (t, 1H, NH). MS (ESI) *m/z* 206 [$(M + 1)^+$].

***trans*-3-(4-Fluorobenzylcarbamoyl)propenoic Acid Ethyl Ester (EtOOCCH=CHCONH-CH$_2$Ph-4-F)** was obtained by mixed anhydride coupling of

equimolar amounts of monoethyl fumarate and 4-fluorobenzylamine to give a pink solid (66% yield). ^{1}H-NMR ($CDCl_3$): 1.32 (t, 3H, CH_2CH_3), 4.21 (q, 2H, CH_2CH_3), 4.47 (d, 2H, N-CH_2-Ph), 6.52-6.56 (d, 1H, J = 15.2 Hz, CH=CHCON), 6.94-6.98 (d, 1H, J = 15.6 Hz, CH=CHCON), 6.99-7.04 (m, 2H, Ph), 7.20-7.27 (m, 2H, Ph).

***trans*-3-(4-Fluorobenzylcarbamoyl)propenoic Acid (16d, HOOCCH=CHCONH-CH_2Ph-4-F).** EtOOCCH=CHCONH-CH_2Ph-4-F was hydrolyzed in MeOH using NaOH (1 M aqueous, 1.1 eq) under standard deblocking conditions to give a clear, colorless syrup (45% yield). ^{1}H-NMR (DMSO-d_6): 4.34-4.35 (d, 2H, N-CH_2-Ph), 6.52-6.55 (d, 1H, J = 15.2 Hz, CH=CHCON), 6.93-6.94 (d, 1H, J = 15.6 Hz, CH=CHCON), 7.14-7.16 (t, 2H, Ph), 7.27-7.31 (t, 2H, Ph), 8.99 (t, 1H, NH).

***trans*-3-Phenylethylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH=CHCONHCH$_2$CH$_2$Ph)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and phenylethylamine to give a clear colorless syrup (78% yield).

***trans*-3-Phenylethylcarbamoylpropenoic Acid (16e, HOOCCH=CHCONHCH$_2$CH$_2$Ph).** EtOOCCH=CHCONHCH$_2$CH$_2$Ph was hydrolyzed in MeOH using NaOH (1 M aqueous, 1.1 eq) under standard deblocking conditions to give a clear, colorless syrup (81% yield). ^{1}H-NMR (DMSO-d_6): 3.54 (t, 2H, N-CH_2-CH_2-Ph), 3.61 (t, 2H, N-CH_2-CH_2-Ph) 6.43-6.47 (d, 1H, J = 15.2 Hz, CH=CHCON), 6.98-7.02 (d, 1H, J = 15.6 Hz, CH=CHCON), 7.14-7.31 (m, 5H, Ph).

***trans*-3-Phenylpropylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH=CHCONHCH$_2$CH$_2$CH$_2$Ph)** was obtained by mixed anhydride coupling of

equimolar amounts of monoethyl fumarate and 3-phenyl-1-propylamine. Purification by column chromatography using 1:1 EtOAc:hexane gave a white powder (80% yield).

***trans*-3-Phenylpropylcarbamoylpropenoic Acid (16f, HOOCCH=CHCONHCH$_2$CH$_2$CH$_2$Ph).** EtOOCCH=CHCONHCH$_2$CH$_2$CH$_2$Ph was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 eq) under standard deblocking conditions to give a white powder (81% yield). ^{1}H-NMR (DMSO-d$_6$): 1.68-1.76 (m, 2H, NH-CH$_2$-*CH$_2$*-CH$_2$-Ph), 2.49-2.59 (t, 2H, NH-CH$_2$-CH$_2$-*CH$_2$*-Ph), 3.12-3.17 (q, 2H, NH-*CH$_2$*-CH$_2$-CH$_2$-Ph) 6.47-6.51 (d, 1H, *J* = 15.6 Hz, *CH*=CHCON), 6.90-6.94 (d, 1H, *J* = 15.6 Hz, CH=*CH*CON), 7.15-7.28 (m, 5H, Ph), 8.51 (t, 1H, NH).

***trans*-3-(1-Naphthylmethylcarbamoyl)propenoic Acid Ethyl Ester (EtOOCCH=CH-CONHCH$_2$-1-Napth)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and naphthalen-1-ylmethanamine. Purification by column chromatography using 2:1 Hexanes:EtOAc to give white powder (86% yield). ^{1}H-NMR (CDCl$_3$): 1.20 (t, 3H, CH$_3$), 3.99 (q, 2H, CH$_2$), 4.93 (d, 2H, *CH$_2$*-naphth), 6.84 (d, 1H, *CH*=CHCON), 6.90 (d. 1H, CH=*CH*CON), 7.38-7.44 (m, 2H, naphthyl), 7.47-7.54 (m, 2H, naphthyl), 7.79 (d, 1H, naphthyl), 7.84 (d, 1H, naphthyl), 7.95 (d, 1H, naphthyl).

***trans*-3-(1-Naphthylmethylcarbamoyl)propenoic Acid (16g, HOOCCH=CH-CONHCH$_2$-1-Napth).** EtOOCCH=CHCONHCH$_2$-1-napth was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 eq) under standard deblocking conditions to give a white solid (76% yield). ^{1}H-NMR (DMSO-d$_6$): 4.82 (d, 2H, *CH$_2$*-naphth), 6.56-6.60 (d, 1H, *J* = 15.6 Hz, C*H*=CHCON), 6.91-6.95 (d. 1H, , *J* = 15.6 Hz, CH=*CH*CON), 7.40-7.57 (m,

4H, naphthyl), 7.84-7.86 (m, 1H, naphthyl), 7.94 (d, 1H, naphthyl), 8.03 (d, 1H, naphthyl), 9.00 (t, 1H, NH).

***trans*-3-(Methyl-1-naphthylmethylcarbamoyl)propenoic Acid Ethyl Ester (EtOOCCH=CHCON(CH_3)CH_2-1-Napth)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and N-methyl-1-naphthylmethylamine hydrochloride. Purification by column chromatography using 1:1 EtOAc:hexane gave a white powder (62% yield). ^{1}H-NMR ($CDCl_3$): 1.29 (t, 3H, CH_3), 3.01 (s, 3H, CH_3), 5.11 (s, 2H, CH_2), 6.87-6.92 (d, 1H, *CH*=CHCON), 7.40-7.52 (m, 5H, CH=*CH*CON and naphthyl), 7.78-7.89 (m, 3H, naphthyl).

***trans*-3-(Methyl-1-naphthylmethylcarbamoyl)propenoic Acid (16h, HOOCCH=CHCON(CH_3)CH_2-1-Napth).** EtOOCCH=CHCON(CH_3)CH_2-1-Napth was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 eq) under standard deblocking conditions to give a white solid (13% yield). ^{1}H-NMR (DMSO-d_6): 3.01 (s, 3H, CH_3), 5.01 (s, 2H, CH_2), 6.61-6.65 (d, 1H, J = 15.2 Hz, *CH*=CHCON), 7.17-7.21 (d, 1H, CH=*CH*CON), 7.37-7.60 (m, 4H, naphthyl), 7.85-8.01 (m, 3H, naphthyl).

***trans*-3-Dibenzylcarbamoylpropenoic Acid Ethyl Ester (EtOOCCH=CHCON($CH_2Ph)_2$)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and dibenzylamine to give a clear, pink syrup (87% yield).

***trans*-3-Dibenzylcarbamoylpropenoic Acid (16j, HOOCCH=CHCON($CH_2Ph)_2$).** EtOOCCH=CHCON($CH_2Ph)_2$ was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 eq) under standard deblocking conditions to give a white powder (91% yield). ^{1}H-NMR (DMSO-d_6): 4.57 (s, 2H, N*CH*$_2$Ph), 4.65 (s, 2H,

N*CH*$_2$Ph), 6.61-6.65 (d, 1H, *CH*=CHCON), 7.15-7.17 (d, 1H, CH=*CH*CON), 7.25-7.50 (m, 10H, 2 x Ph).

***trans*-3-(2-(1H-indol-3-yl)ethyl)carbamoylpropenoic Acid Ethyl Ester (EtOOCCH=CH-CONH(CH$_2$)$_2$indole)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and 2-(1H-indol-2-yl)ethylamine to give a brown solid (64% yield).

***trans*-3-(2-(1H-indol-3-yl)ethyl)carbamoylpropenoic Acid (16i, HOOCCH=CH-CONH(CH$_2$)$_2$indole).** EtOOCCH=CH-CONH(CH$_2$)$_2$indole was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 eq) under standard deblocking conditions to give a off-white solid (87% yield). ^{1}H-NMR (DMSO-d$_6$): 2.47 (t, 1H, CH), 2.84-2.87 (t, 2H, CH$_2$), 3.41-3.46 (t, 2H, CH$_2$), 6.49-6.53 (d, 1H, *CH*=CHCON), 6.89-6.93 (d, 1H, CH=*CH*CON), 6.96-7.06 (m, 2H, 2 x CH), 7.14 (s, 1H, CH), 7.31 (d, 1H, CH), 7.51 (d, 1H, CH), 8.60 (t, 1H, NH), 10.81 (s, 1H, COOH).

***trans*-3-(3,3-diphenylpropyl)carbamoylpropenoic Acid Ethyl Ester (EtOOCCH=CH-CONH(CH$_2$)$_2$CH(Ph)$_2$)** was obtained by mixed anhydride coupling of equimolar amounts of monoethyl fumarate and 3,3-diphenylpropan-1-amine to give a yellow oil (74% yield).

***trans*-3-(3,3-diphenylpropyl)carbamoylpropenoic Acid (16k, HOOCCH=CH-CONH(CH$_2$)$_2$CH(Ph)$_2$).** EtOOCCH=CH-CONH(CH$_2$)$_2$CH(Ph)$_2$ was hydrolyzed in EtOH using NaOH (1 M aqueous, 1.1 eq) under standard deblocking conditions to give a white solid (83% yield). ^{1}H-NMR (DMSO-d$_6$): 2.18-2.30 (m, 2H, CH$_2$), 3.02-3.07 (m, 2H, CH$_2$), 3.99 (t, 1H, CH), 6.47-6.51 (d, 1H, *CH*=CHCON), 6.90-6.94 (d, 1H, CH=*CH*CON), 7.12-7.30 (m, 10H, 2 x Ph), 8.53 (t, 1H, NH).

General Procedure for Coupling of the Mono Ethyl Ester Epoxysuccinates to Amines. The method used was the mixed anhydride coupling method. The mono ethyl ester epoxysuccinate (1 eq) was dissolved in CH_2Cl_2 and cooled to -20 °C. To the reaction mixture was added NMM (3 eq) and then iBCF (3 eq). The reaction mixture was stirred at -20 °C for 15-20 minutes and then the amine (3 eq) was added. The reaction mixture was then stirred at -20 °C for one hour and then at room temperature overnight. The solvent was removed, and the crude product was dissolved in EtOAc. The organic layer was then washed with of 2% citric acid, saturated $NaHCO_3$ and brine. The product was purified with column chromatography as needed. Hydrolysis of the ethyl ester with 1 M NaOH (1.5 eq) in EtOH gave the desired amides.

(*2S,3S*)-Oxirane-2,3-dicarboxylic Acid Dibenzyl Amide (18m, HOOC-EP-CON($CH_2Ph)_2$) was obtained by mixed anhydride coupling of the mono ethyl ester epoxysuccinate and dibenzylamine to give a clear oil and then hydrolyzed in basic conditions to the corresponding acid. ^{1}H NMR (DMSO-d_6): 3.51 (d, 1H, epoxy CH), 4.03 (d, 1H, epoxy CH), 4.50 (dd, 2H, NCH_2Ph), 4.70 (dd, 2H, NCH_2Ph), 7.18-7.36 (m, 10H, 2 x Ph).

(*2S,3S*)-Oxirane-2,3-dicarboxylic Acid 1-Naphthylmethyl Amide (18l, HOOC-EP-CONH-1-CH_2-Napth) was obtained by mixed anhydride coupling of the mono ethyl ester epoxysuccinate and 1-naphthylmethylamine to give a yellow powder and then hydrolyzed in basic conditions to the corresponding acid. ^{1}H NMR (Acetone-d_6): 3.59 (d, 1H, epoxy CH), 3.82 (d, 1H, epoxy CH), 5.21-5.36 (m, 4H, N(1-CH_2-naphthyl)$_2$), 7.37-7.56 (m, 8H, N(1-CH_2- naphthyl)$_2$), 7.84-7.96 (m, 5H, N(1-CH_2-naphthyl)$_2$), 8.15-8.18 (m, 1H, N(1-CH_2- naphthyl)$_2$).

General Procedure for the Synthesis of Aza-Peptide Michael Acceptors and Aza-Peptide Epoxides by the HOBt/EDC Coupling Method. To a stirred solution of the fumaric acid or epoxide precursor (1.5 eq) in DMF at -10 °C was added HOBt (1.5 eq), the peptidyl hydrazide precursor (1 eq) and EDC (1.5 eq) was added. The mixture was allowed to react for 16 h at room temperature. The DMF was evaporated, and the residue was redissolved in EtOAc. The organic layer was washed with 2% citric acid, saturated $NaHCO_3$, saturated NaCl, dried over $MgSO_4$, and concentrated. Column chromatography on silica gel afforded the aza-peptidyl fumarate and aza-peptidyl epoxide derivatives.

N^2-(N-Benzyloxycarbonylleucyl)-N^1-ethyl-N^1-*trans*-(3-ethoxycarbonylpropenoyl)hydrazine (19a, Cbz-Leu-AAbu-CH=CH-COOEt). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 10% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (20% yield). ^{1}H-NMR ($CDCl_3$): 0.92-1.07 (m, 9H, 2 x Leu-CH_3 and Abu-CH_3), 1.26 (t, 3H, CH_3), 1.56-1.73 (m, 3H, Leu-CH and CH_2), 3.66 (m, 2H, CH_2), 4.15-4.25 (m, 3H, α-H and CH_2), 5.12 (s, 2H, CH_2), 5.31 (d, 1H, NH), 6.78-6.82 (d, 1H, *CH*=CHCON), 7.20-7.24 (d, 1H, CH=*CH*CON), 7.33 (m, 5H, Ph), 8.86 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{22}H_{32}N_3O_6$: 434.2291. Observed *m/z* 434.2287. Anal. Calcd. for $C_{22}H_{31}N_3O_6 \cdot 0.15H_2O$: C, 60.58; H, 7.23; N, 9.63. Found: C, 60.59; H, 7.31; N, 9.61.

N^2-(N-Benzyloxycarbonylleucyl)-N^1-ethyl-N^1-*trans*-(3-(methyl-N-(1-naphthylmethyl)carbamoyl)propenoyl)hydrazine (19h, Cbz-Leu-AAbu-CH=CH-CON(CH_3)CH_2-1-naphthyl). This compound was obtained using the HOBt/EDC

coupling method and purified by column chromatography using 5% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (41% yield). ^{1}H-NMR (DMSO-d_6): 0.85-1.06 (m, 9H, 2 x Leu-CH_3 and Abu-CH_3), 1.46-1.64 (m, 3H, Leu-CH and CH_2), 2.97 (s, 3H, CH_3), 3.27 (s, 1H, CH), 3.74 (s, 1H, CH), 4.08 (m, 1H, α-H), 5.04-5.18 (m, 4H, 2 x CH_2), 7.11-7.70 (m, 12H, Ph, naphthyl, *CH*=CHCON, CH=*CH*CON and NH), 7.85-8.05 (m, 3H, naphthyl), 10.76 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{32}H_{39}N_4O_5$: 559.2921. Observed *m/z* 559.2896. Anal. Calcd. for $C_{32}H_{38}N_4O_5 \cdot 0.2H_2O$: C, 68.36; H, 6.88; N, 9.96. Found: C, 68.28; H, 6.93; N, 9.91.

N^2-(4-Morpholinylcarbonylphenylalanyl)-N^1-phenylethyl-N^1-*trans*-(3-(N-methyl-N-phenylcarbamoyl)propenoyl)hydrazine (20b, Mu-Phe-AHph-CH=CH-CON(CH_3)Ph). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (28% yield). ^{1}H-NMR (DMSO-d_6): 2.92-2.98 (m, 2H, *CH_2*CH_2Ph), 3.20-3.48 (m, 15H, morpholine, CH_2*CH_2*Ph and CH_2Ph and CH_3), 4.38 (m, 1H, α-H), 6.55-6.59 (d, 1H, *CH*=CHCON), 6.90 (d, 1H, NH), 7.04-7.46 (m, 16H, 3 x Ph and CH=*CH*CON), 10.75 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{33}H_{38}N_5O_5$: 584.2867. Observed *m/z* 584.2868. Anal. Calcd. for $C_{31}H_{38}N_6O_7 \cdot 0.5$EtOAc: C, 66.97; H, 6.58; N, 11.16. Found: C, 67.45; H, 6.44; N, 11.05.

N^2-(4-Morpholinylcarbonylphenylalanyl)-N^1-phenylethyl-N^1-*trans*-(3-benzylcarbamoylpropenoyl)hydrazine (20c, Mu-Phe-AHph-CH=CH-CONHCH$_2$Ph). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (24% yield). ^{1}H-NMR

(DMSO-d_6): 2.10-2.14 (m, 7H, piperazine and NCH_3), 2.95 (m, 2H, *CH_2*CH_2Ph), 3.22-3.44 (m, 8H, morpholine, CH_2*CH_2*Ph and CH_2Ph), 4.34 (m, 3H, CH_2Ph and α-H), 6.85-6.89 (m, 2H, *CH*=CHCON and NH), 7.13-7.29 (m, 16H, 3 x Ph and CH=*CH*CON), 8.89 (s, 1H, NH), 10.75 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{33}H_{38}N_5O_5$: 584.2880. Observed *m/z* 584.2867. Anal. Calcd. for $C_{33}H_{37}N_5O_5 \cdot 0.4H_2O$: C, 67.08; H, 6.45; N, 11.58. Found: C, 67.07; H, 6.39; N, 11.56.

N^2-(4-Morpholinylcarbonylphenylalanyl)-N^1-phenylethyl-N^1-*trans*-(3-(3,3-diphenylpropyl)carbamoylpropenoyl)hydrazine (20k, Mu-Phe-AHph-CH=CH-CONH(CH_2)$_2$CH(Ph)$_2$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (46% yield). ^{1}H-NMR (DMSO-d_6): 2.16-2.20 (m, 2H, CH_2), 2.89-2.99 (m, 4H, CH_2Ph and CH_2), 3.23-3.32 (m, 4H, morpholine, CH_2*CH_2*Ph and CH_2Ph), 3.44 (s, 4H, morpholine), 3.95-3.99 (t, 1H, CH), 4.35 (m, 1H, α-H), 6.79-6.88 (m, 2H, *CH*=CHCON and NH), 7.05-7.26 (m, 21H, 4 x Ph and CH=*CH*CON), 8.44 (s, 1H, NH), 10.75 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{41}H_{46}N_5O_5$: 688.3493. Observed *m/z* 688.3461. Anal. Calcd. for $C_{41}H_{45}N_5O_5 \cdot 0.3H_2O$: C, 71.04; H, 6.63; N, 10.10. Found: C, 71.07; H, 6.58; N, 9.67.

(*2S,3S*)-2-(1-Naphthylmethylcarbamoyl)-3-(N^2-(N-4-Morpholinylcarbonylphenylalanyl)-N^1-phenylethylhydrazinocarbonyl)oxirane (22l, Mu-Phe-AHph-EP(*S,S*)-CONHCH_2-1-naphthyl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (30% yield). ^{1}H-NMR (DMSO-d_6): 2.94 (m, 2H, *CH_2*CH_2Ph), 3.20-3.53 (m, 12H,

morpholine, CH_2CH_2Ph and CH_2Ph), 3.72 (s, 1H, epoxy-CH), 4.04 (s, 1H, epoxy-CH), 4.31 (m, 1H, α-H), 4.72 (m, 2H, CH_2), 6.93 (d, 1H, NH), 7.18-7.52 (m, 14H, 2 x Ph, naphthyl), 7.82-7.98 (m, 3H, naphthyl), 8.94 (s, 1H, NH), 10.78 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{37}H_{40}N_5O_6$: 650.2979. Observed *m/z* 650.2993. Anal. Calcd. for $C_{37}H_{39}N_5O_6 \cdot 0.5H_2O$: C, 67.46; H, 6.12; N, 10.63. Found: C, 67.36; H, 6.07; N, 10.51.

(*2S,3S*)-2-(Dibenzylcarbamoyl)-3-(N^2-(N-4-Morpholinylcarbonylphenylalanyl)-N^1-phenylethylhydrazinocarbonyl)oxirane (22m, Mu-Phe-AHph-EP(*S,S*)-CON($CH_2Ph)_2$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (20% yield). ^{1}H-NMR (DMSO-d_6): 2.99 (m, 2H, CH_2CH_2Ph), 3.23-3.46 (m, 12H, morpholine, CH_2CH_2Ph and CH_2Ph), 3.72 (s, 1H, epoxy-CH), 3.87 (s, 1H, epoxy-CH), 4.03 (m, 1H, α-H), 4.222-4.70 (m, 4H, 2 x CH_2Ph), 6.95 (d, 1H, NH), 7.18-7.36 (m, 20H, 4 x Ph), 10.75 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{40}H_{44}N_5O_6$: 690.3292. Observed *m/z* 690.3281. Anal. Calcd. for $C_{40}H_{43}N_5O_6 \cdot 0.7H_2O$: C, 68.40; H, 6.37; N, 9.97. Found: C, 68.43; H, 6.32; N, 9.82.

N^2-(4-Methylpiperazinylcarbonylphenylalanyl)-N^1-phenylethyl-N^1-*trans*-(3-(N-methyl-N-phenylcarbamoyl)propenoyl)hydrazine (21b, Me-Piz-Phe-AHph-CH=CH-CON(CH_3)Ph). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (16% yield). ^{1}H-NMR (DMSO-d_6): ^{1}H-NMR (DMSO-d_6): 2.10-2.14 (m, 7H, piperazine and NCH_3), 2.96-2.98 (m, 2H, CH_2CH_2Ph), 3.23-3.27 (m, 8H, piperazine, CH_2CH_2Ph and CH_2Ph and CH_3),

4.35 (m, 1H, α-H), 6.55-6.59 (d, 1H, *CH*=CHCON), 6.83 (d, 1H, NH), 7.13-7.45 (m, 16H, 3 x Ph and CH=*CH*CON), 10.73 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{34}H_{41}N_6O_4$: 597.3184. Observed *m/z* 597.3184. Anal. Calcd. for $C_{34}H_{40}N_6O_4 \cdot 0.65H_2O$: C, 67.12; H, 6.84; N, 13.81. Found: C, 67.08; H, 6.75; N, 13.82.

N^2-(4-Methylpiperazinylcarbonylphenylalanyl)-N^1-phenylethyl-N^1-*trans*-(3-benzylcarbamoylpropenoyl)hydrazine (21c, Me-Piz-Phe-AHph-CH=CH-$CONHCH_2Ph$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (11% yield). ^{1}H-NMR (DMSO-d_6): 2.10-2.14 (m, 7H, piperazine and NCH_3), 2.95 (m, 2H, CH_2CH_2Ph), 3.24-3.33 (m, 8H, piperazine, CH_2CH_2Ph and CH_2Ph), 4.35 (m, 3H, CH2Ph and α-H), 6.84-6.88 (m, 2H, *CH*=CHCON and NH), 7.18-7.29 (m, 16H, 3 x Ph and CH=*CH*CON), 8.89 (s, 1H, NH), 10.74 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{34}H_{41}N_6O_4$: 597.3189. Observed *m/z* 597.3229. Anal. Calcd. for $C_{34}H_{40}N_6O_4 \cdot 1.1H_2O$: C, 66.24; H, 6.90; N, 13.63. Found: C, 66.22; H, 6.77; N, 13.55.

N^2-(4-Methylpiperazinylcarbonylphenylalanyl)-N^1-phenylethyl-N^1-*trans*-(3-(4-fluorobenzyl)carbamoylpropenoyl)hydrazine (21d, Me-Piz-Phe-AHph-CH=CH-$CONHCH_2Ph$-4-F). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (23% yield). ^{1}H-NMR (DMSO-d_6): 2.10-2.14 (m, 7H, piperazine and NCH_3), 2.95 (m, 2H, CH_2CH_2Ph), 3.24-3.32 (m, 8H, piperazine, CH_2CH_2Ph and CH_2Ph), 4.33 (m, 3H, CH_2Ph and α-H), 6.83-6.87 (m, 2H, *CH*=CHCON and NH), 7.09-7.28 (m, 15H, 2 x Ph, Ph-F and CH=*CH*CON),

8.90 (s, 1H, NH), 10.74 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{34}H_{40}N_6O_4F$: 615.3095. Observed *m/z* 615.3136. Anal. Calcd. for $C_{34}H_{39}N_6O_4F \cdot 0.5H_2O$: C, 65.47; H, 6.46; N, 13.47. Found: C, 65.54; H, 6.41; N, 13.45.

N^2-(4-Methylpiperazinylcarbonylphenylalanyl)-N^1-phenylethyl-N^1-*trans*-(3-phenylethylcarbamoylpropenoyl)hydrazine (21e, Me-Piz-Phe-AHph-CH=CH-CONH(CH_2)$_2$Ph). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (23% yield). ^{1}H-NMR (DMSO-d_6): 2.11-2.17 (m, 7H, piperazine and NCH_3), 2.70-2.74 (t, 2H, CH_2CH_2Ph), 2.95 (m, 2H, CH_2CH_2Ph), 3.27-3.32 (m, 10H, piperazine, CH_2CH_2Ph, CH_2Ph and CH_2CH_2Ph), 4.34 (m, 1H, α-H), 6.82 (m, 2H, *CH*=CHCON and NH), 7.07-7.30 (m, 16H, 3 x Ph and CH=*CH*CON), 8.49 (s, 1H, NH), 10.73 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{35}H_{43}N_6O_4$: 611.3346. Observed *m/z* 611.3361. Anal. Calcd. for $C_{35}H_{42}N_6O_4 \cdot 0.15H_2O$: C, 68.53; H, 6.95; N, 13.70. Found: C, 68.55; H, 6.79; N, 13.53.

N^2-(4-Methylpiperazinylcarbonylphenylalanyl)-N^1-phenylethyl-N^1-*trans*-(3-phenylpropylcarbamoylpropenoyl)hydrazine (21f, Me-Piz-Phe-AHph-CH=CH-CONH(CH_2)$_3$Ph). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (15% yield). ^{1}H-NMR (DMSO-d_6): 1.67-1.74 (m, 2H, $CH_2CH_2CH_2$), 2.10-2.15 (m, 7H, piperazine and NCH_3), 2.54-2.58 (t, 2H, $CH_2CH_2CH_2Ph$), 2.96 (m, 2H, CH_2CH_2Ph), 3.12-3.32 (m, 10H, piperazine, CH_2CH_2Ph, CH_2Ph and $CH_2CH_2CH_2Ph$), 4.32 (m, 1H, α-H), 6.81-6.84 (m, 2H, *CH*=CHCON and NH), 7.07-7.29 (m, 16H, 3 x Ph and CH=*CH*CON), 8.42 (s, 1H,

NH), 10.73 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{36}H_{45}N_6O_4$: 625.3458. Observed *m/z* 625.3463. Anal. Calcd. for $C_{36}H_{44}N_6O_4 \cdot 0.8H_2O$: C, 67.65; H, 7.19; N, 13.15. Found: C, 67.66; H, 6.92; N, 13.11.

N^2-(4-Methylpiperazinylcarbonylphenylalanyl)-N^1-phenylethyl-N^1-*trans*-(3-(1-naphthylmethylcarbamoyl)propenoyl)hydrazine (21g, Me-Piz-Phe-AHph-CH=CH-CONHCH$_2$-1-naphthyl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (28% yield). ^{1}H-NMR (DMSO-d_6): 2.08-2.13 (m, 7H, piperazine and NCH_3), 2.97 (m, 2H, *CH_2*CH_2Ph), 3.23-3.32 (m, 8H, piperazine, CH_2*CH_2*Ph and CH_2Ph), 4.33 (m, 1H, α-H), 4.81 (d, 2H, CH_2), 6.84-6.91 (m, 2H, *CH*=CHCON and NH), 7.18-7.53 (m, 15H, 2 x Ph, naphthyl and CH=*CH*CON), 7.83-8.02 (m, 3H, naphthyl), 8.93 (s, 1H, NH), 10.76 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{38}H_{43}N_6O_4$: 647.3340. Observed *m/z* 647.3249. Anal. Calcd. for $C_{38}H_{42}N_6O_4 \cdot 0.5H_2O$: C, 69.79; H, 6.60; N, 12.85. Found: C, 69.64; H, 6.50; N, 12.41.

N^2-(4-Methylpiperazinylcarbonylphenylalanyl)-N^1-phenylethyl-N^1-*trans*-(3-(methyl-1-naphthylmethylcarbamoyl)propenoyl)hydrazine (21h, Me-Piz-Phe-AHph-CH=CH-CON(CH$_3$)CH$_2$-1-naphthyl). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (12% yield). ^{1}H-NMR (DMSO-d_6): 2.11-2.19 (m, 7H, piperazine and NCH_3), 2.96-2.99 (m, 2H, *CH_2*CH_2Ph and CH_3), 3.27-3.33 (m, 8H, piperazine, CH_2*CH_2*Ph, CH_2Ph), 4.34 (m, 1H, α-H), 5.01-5.17 (m, 2H, CH2), 6.86 (d, 1H, NH), 7.10-7.59 (m, 16H, 2 x Ph,

naphthyl, *CH*=CHCON and CH=*CH*CON), 7.84-8.06 (m, 3H, naphthyl), 10.74 (d, 1H, NH). HRMS (FAB) Calcd. for $C_{39}H_{45}N_6O_4$: 661.3497. Observed *m/z* 661.3486. Anal. Calcd. for $C_{39}H_{44}N_6O_4 \cdot 0.75H_2O$: C, 69.47; H, 6.80; N, 12.46. Found: C, 69.41; H, 6.77; N, 12.35.

N^2-(4-Methylpiperazinylcarbonylphenylalanyl)-N^1-phenylethyl-N^1-*trans*-(3-(2-(1H-indol-3-yl)ethyl)carbamoyl)propenoylhydrazine (21i, Me-Piz-Phe-AHph-CH=CH-CONH$(CH_2)_2$indole). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (32% yield). ^{1}H-NMR (DMSO-d_6): 2.10-2.17 (m, 7H, piperazine and NCH_3), 2.82-2.86 (t, 2H, CH_2), 2.98 (m, 2H, CH_2CH_2Ph), 3.28-3.43 (m, 10H, piperazine, CH_2CH_2Ph, CH_2Ph and CH_2), 4.35 (m, 1H, α-H), 6.84-7.51 (m, 18H, 2 x Ph, indole, *CH*=CHCON, CH=*CH*CON and NH), 8.54 (s, 1H, NH), 10.75-10.81 (m, 2H, 2 x NH). HRMS (FAB) Calcd. for $C_{37}H_{44}N_7O_4$: 650.3455. Observed *m/z* 650.3473. Anal. Calcd. for $C_{37}H_{43}N_7O_4 \cdot 0.9H_2O$: C, 66.73; H, 6.78; N, 14.72. Found: C, 66.76; H, 6.67; N, 14.53.

N^2-(4-Methylpiperazinylcarbonylphenylalanyl)-N^1-phenylethyl-N^1-*trans*-(3-dibenzylcarbamoyl)propenoylhydrazine (21j, Me-Piz-Phe-AHph-CH=CH-CON$(CH_2Ph)_2$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (34% yield). ^{1}H-NMR (DMSO-d_6): 2.10-2.14 (m, 7H, piperazine and NCH_3), 2.97 (m, 2H, CH_2CH_2Ph), 3.26-3.33 (m, 8H, piperazine, CH_2CH_2Ph and CH_2Ph), 4.34 (m, 1H, α-H), 4.48-4.59 (m, 4H, 2 x CH_2Ph), 6.85 (d, 1H, NH), 7.14-7.37 (m, 22H, 4 x Ph, *CH*=CHCON and

CH=*CH*CON), 8.89 (s, 1H, NH), 10.75 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{41}H_{47}N_6O_4$: 687.3659. Observed *m/z* 687.3662. Anal. Calcd. for $C_{41}H_{46}N_6O_4 \cdot 0.3H_2O$: C, 71.14; H, 6.78; N, 12.14. Found: C, 71.19; H, 6.69; N, 12.10.

N^2-(4-Methylpiperazinylcarbonylphenylalanyl)-N^1-phenylethyl-N^1-*trans*-(3-(3,3-diphenylpropyl)carbamoyl)propenoylhydrazine (21k, Me-Piz-Phe-AHph-CH=CH-CONH$(CH_2)_2$CH$(Ph)_2$). This compound was obtained using the HOBt/EDC coupling method and purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (19% yield). ^{1}H-NMR (DMSO-d_6): 2.09-2.19 (m, 9H, piperazine, NCH_3 and CH_2), 2.94-2.99 (m, 4H, *CH_2*CH_2Ph and CH_2), 3.23-3.32 (m, 8H, piperazine, CH_2*CH_2*Ph, CH_2Ph and CH_2), 3.95-3.99 (t, 1H, CH), 4.32 (m, 1H, α-H), 6.79-6.83 (m, 2H, *CH*=CHCON and NH), 7.06-7.26 (m, 21H, 4 x Ph and CH=*CH*CON), 8.43 (s, 1H, NH), 10.71 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{42}H_{49}N_6O_4$: 701.3810. Observed *m/z* 701.3796. Anal. Calcd. for $C_{42}H_{48}N_6O_4 \cdot 0.2H_2O$: C, 71.61; H, 6.92; N, 11.93. Found: C, 71.51; H, 6.94; N, 11.83.

CHAPTER 4

DESIGN, SYNTHESIS AND EVALUATION OF PEPTIDYL KETOAMIDES, AMINOKETONES AND AZA-PEPTIDYL KETONES AS CALPAIN INHIBITORS

INTRODUCTION

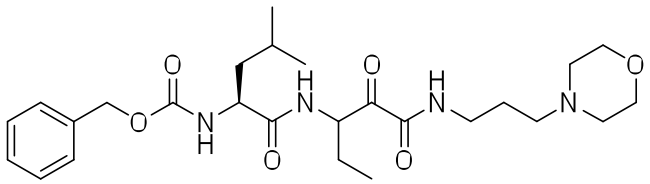
Calpains are cysteine proteases that require calcium for activation. They belong to Clan CA of cysteine proteases together with cruzain, rhodesain, papain and cathepsins. There are multiple isoforms of calpain that are both ubiquitous and tissue specific. Calpain I (μ-calpain) and calpain II (m-calpain) are the two major calpain isoforms that are widely distributed in mammalian cells. These two isoforms are very similar but differ in the calcium concentration that they require to become activated. Calpain I is activated by micromolar concentration of Ca^{+2} whereas calpain II is activated by millimolar concentration of Ca^{+2}. Calpains are involved in a variety of calcium-regulated biological processes, such as cell proliferation and differentiation, apoptosis, membrane fusion, signal transduction and platelet activation. Enhanced calpain activity has been observed in a number of diseases including ischemic[101, 102] and traumatic[103, 104] brain injury, cancer,[105-107] muscular dystrophy,[108, 109] cataracts,[110] strokes,[111] and neurological disorders like Alzheimer's,[112, 113] Huntington's,[114] and Parkinson's[115, 116] diseases and multiple sclerosis.[117, 118] Involvement of calpains in a wide variety of biological processes and diseases makes them an important target for the development of inhibitors.

Synthetic calpain inhibitors can be divided into two groups: peptidic inhibitors and non-peptidic inhibitors. Peptidic inhibitors can further be divided into two groups: reversible inhibitors and irreversible inhibitors. Peptidyl aldehydes,[119-121] α-ketoacids,[95] α-ketoesters,[95] α-ketoamides,[94, 95] α-diketones[122] and α-keto phosphorus[123, 124] are examples of reversible peptidyl inhibitors whereas peptidyl epoxysuccinates,[125-127] vinyl sulfones,[96] acyloxymethyl ketones, diazomethyl ketones,[128] chloromethyl ketones[129] are examples of irreversible peptidyl inhibitors of calpain. Reversible inhibitors of calpain are favored over the irreversible inhibitors for drug development since there are many isoforms of calpains and nonspecific inhibition of these isoforms can cause severe side effects.

Synthetic calpain inhibitors have been shown to protect against neuronal loss and improve neurological function in animal models of Alzheimer's disease,[130] traumatic brain injury,[131] optic nerve degeneration,[132] spinal cord injury,[133] Taxol-induced sensory neuropathy.[134] The neuroprotective effects of calpain inhibitors have been well established but their use in treatment of human diseases is challenged by their inability to cross the blood-brain barrier. The blood brain barrier (BBB) is a metabolic or cellular structure in the central nervous system (CNS) that restricts the passage of various chemical substances between the bloodstream and the neural tissue itself, while still allowing the passage of substances essential to metabolic function.

Several attempts are reported in the literature to allow the entry of these compounds into central nervous system. In most designs calpain inhibitors were combined structural features recognized by specific transporters in the brain. For example, the calpain inhibitor leupeptin was fused with taurine to facilitate access to the

brain and spinal cord via the taurine transport system[135] and a leupeptin analog was covalently bound to L-aminocarnitine for the penetration of the inhibitor into muscle cells via the carnitine transporter. A series of ketoamide calpain inhibitors were coupled to various muscle cell targeting capping groups to assist the accumulation of the compounds in muscle cells for the treatment of Duchenne Muscular Dystrophy and an improved uptake into muscle cells have been observed.[136]



AK295

Figure 4.1. Cbz-Leu-Abu-CONH-$(CH_2)_3$-morpholinyl (AK295)

AK295 is a reversible α-ketoamide calpain inhibitor and it has been shown that AK295 is effective in the treatment of experimental head trauma,[131] focal brain ischaemia[137] and prevents axonal degeneration caused by axotomy or exposure to vincristine[138] and Taxol.[134] Although the therapeutic efficacy of AK295 has been well established, its development as a drug is hampered by its inability to cross the BBB. We decided to design new analogs of AK295 that can cross the BBB. For the design of AK295 analogs, we decided to incorporate structural features that could be recognized by several BBB transport systems. Thus, we expected that these new calpain inhibitors would be more permeable to the BBB than AK295 and penetration into the brain and spinal cord would provide increased therapeutic efficacy through increased access of the calpain inhibitors to the nervous system.

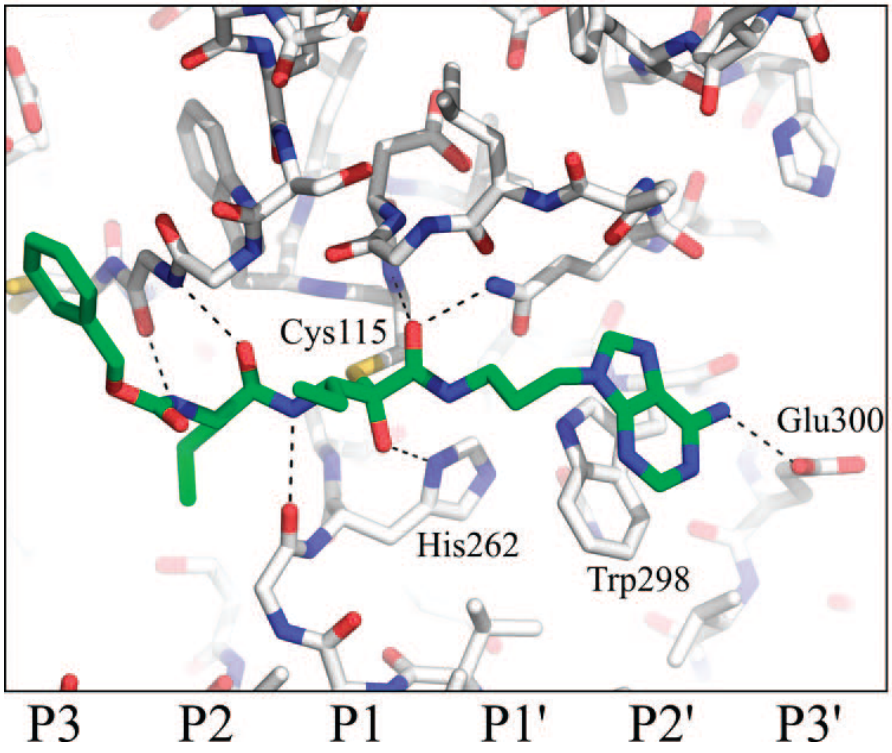
Charged and polar compounds, many of which are essential to the brain, do not cross the BBB and there are multiple transport systems to facilitate the delivery of these compounds to the brain. We have focused on two transport systems; choline and nucleoside transport systems. Choline, a positively charged molecule, has a critical role in the CNS as a precursor to the neurotransmitter acetylcholine. As a charged molecule, choline does not cross the BBB and its uptake into brain is dependent upon carrier-mediated transport. The BBB choline transporter is responsible for the transportation of choline across the BBB and it has been showed that this transporter can be used as a brain drug delivery vector for choline analogs.[139] Nucleosides, the building blocks of RNA and DNA, are hydrophilic compounds and do not readily penetrate cell membranes by passive diffusion. There are several nucleoside transporters for the active transport of these molecules.[140] Nucleoside transporters are sensitive or specific for different heterocyclic bases. It has been shown that several nucleoside reverse transcriptase inhibitors that are used in the treatment of HIV infection are transported into the CNS by these nucleoside transporters.[141]

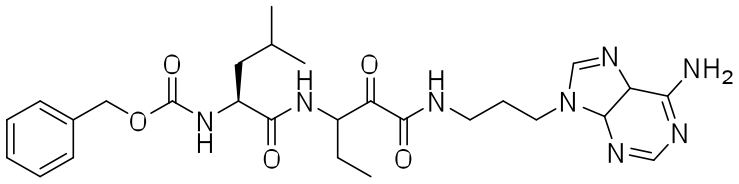
4.1. PEPTIDYL α-KETOAMIDES: POTENT INHIBITORS OF CALPAIN INHIBITOR DESIGN

Peptidyl inhibitors of calpain usually contain an electrophilic warhead that reacts with the active site cysteine to inhibit the enzyme. Among the various kinds of warheads, we focused on the synthesis of α-ketoamides. Peptidyl α-ketoamides inactivate the enzyme by forming a reversible hemithioketal adduct, and therefore minimizing possible side effects which could be observed with irreversible inhibitors. Peptidyl α-ketoamides are also more stable both chemically and metabolically compared to other reversible warheads, such as peptidyl aldehydes.[10] The major problem with α-ketoamides for their development as potential therapeutic targets is their inhibition of other cysteine proteases. Therefore, there is a need for calpain-specific inhibitors that will not affect other cysteine proteases.

A wide variety of α-ketoamides have been synthesized to develop more selective calpain inhibitors by increasing the calpain specificity versus other cysteine proteases. Most of these compounds were designed to explore the interactions in the unprimed side of calpain to enhance selectivity.[142, 143] From these studies, it has been found that phenylalanine and leucine were preferred in the P1 and P2 positions; respectively.[144] However, targeting the unprimed region to improve specificity is not sufficient since there are other cysteine proteases that also prefer these amino acids in P1 and P2 positions.[145] To target calpain specifically, researchers focused on extending the inhibitors into the primed side of calpain.[146, 147] Peptidyl α-ketoamides previously synthesized in our group showed that the extension of the inhibitors into the primed

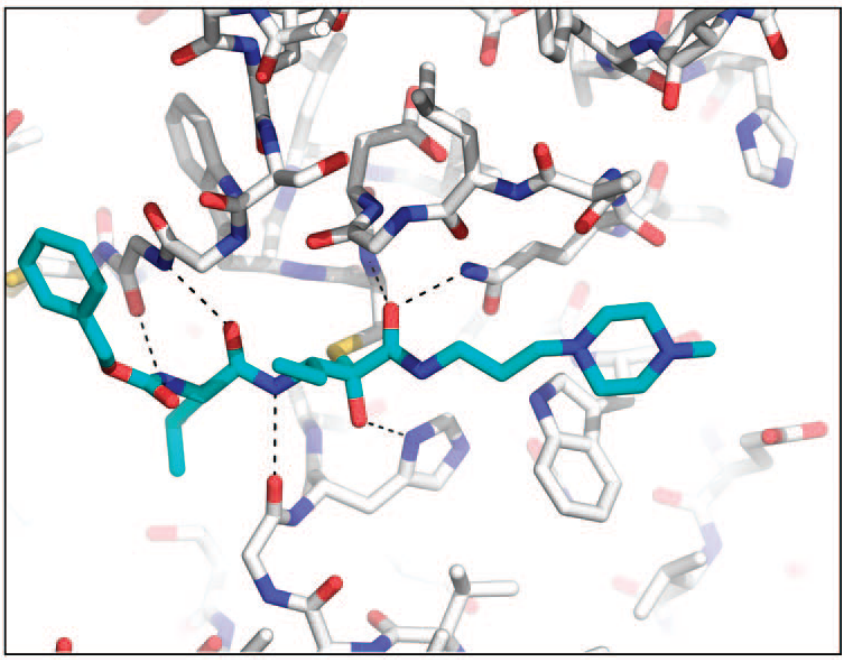
region increased the potency of the inhibitors, and N-monosubstituted peptidyl α-ketoamides were much more potent than the corresponding N,N-disubstituted peptidyl α-ketoamides.

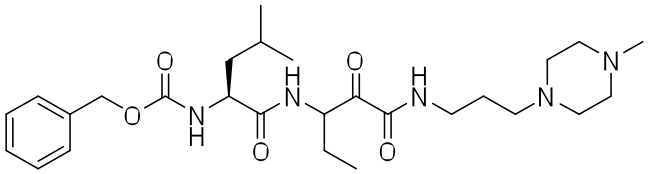




ZLAK3001

Figure 4.1.1. Crystal Structure of ZLAK3001 bound to Calpain I.





ZLAK3002

Figure 4.1.2. Crystal Structure of ZLAK3002 bound to Calpain I.

To synthesize compounds that can cross the BBB and selectivity inhibit calpain, peptidyl α-ketoamides with choline-like structures in the primed side and a peptidyl α-ketoamide with an adenine residue in the primed side were synthesized by Dr. Zhao Zhao Li from our lab. Crystal structures of ZLAK3001 (Figure 4.1.1) and ZLAK3002 (Figure 4.1.2.) bound to rat calpain I protease core (μI-II) were obtained by Campbell et al.[148] It has been demonstrated that the adenine moiety of ZLAK3001 stacks against a tryptophan

in the catalytic site of calpain I and the amino group of adenine is forming a hydrogen bond to Glu300. Neither this stacking nor hydrogen bond formation was observed between the piperazinyl ring of ZLAK3002 and the primed side region. It has been shown that ZLAK3001 and ZLAK3002 penetrate into the brain by highly sensitive and specific HPLC/mass spectrometry assays.

Design of Novel Calpain Inhibitors

A hydrophobic pocket formed by Ala262, Ile263 and Val269 near the C2 carbon of adenine moiety has been observed in the crystal structure of ZLAK3001 bound to calpain I. To facilitate interactions with this hydrophobic pocket, we have synthesized peptidyl α-ketoamides with 2-methoxyadenine moiety in the primed region. We proposed that this modification will increase the potency and selectivity and generate compounds that have the potential to cross the BBB. We have synthesized two peptidyl α-ketoamides with cytosine in the primed region since the cytosine derivative can make the same interactions as observed with ZLAK3001. We have also synthesized an analog of ZLAK3001 where we changed the P1 residue from Abu to Phe, since Phe in P1 position is preferred by calpain.

CHEMISTRY

Synthesis of the peptidyl α-ketoacids are shown in Figure 4.1.3. This chemistry has previously been reported.[149, 150] *N*-benzyloxycarbonylleucine was reacted with aminobutyric acid methyl ester or phenylalanine methyl ester using the HOBt and DCC coupling procedure. The dipeptide methyl esters were hydrolyzed with 1 M NaOH under

standard deblocking conditions. The α-ketoesters were prepared by a two step Dakin-West reaction from the corresponding dipeptide acid. The dipeptide acids were reacted with ethyl oxalyl chloride in the presence of pyridine and 4-dimethylaminopyridine (DMAP) to form peptidyl α-enol esters. The peptidyl α-enol esters were then converted to peptidyl α-ketoesters by reacting with triethylamine. The peptidyl α-ketoacids were obtained by the hydrolysis of the peptidyl α-ketoesters under standard deblocking conditions.

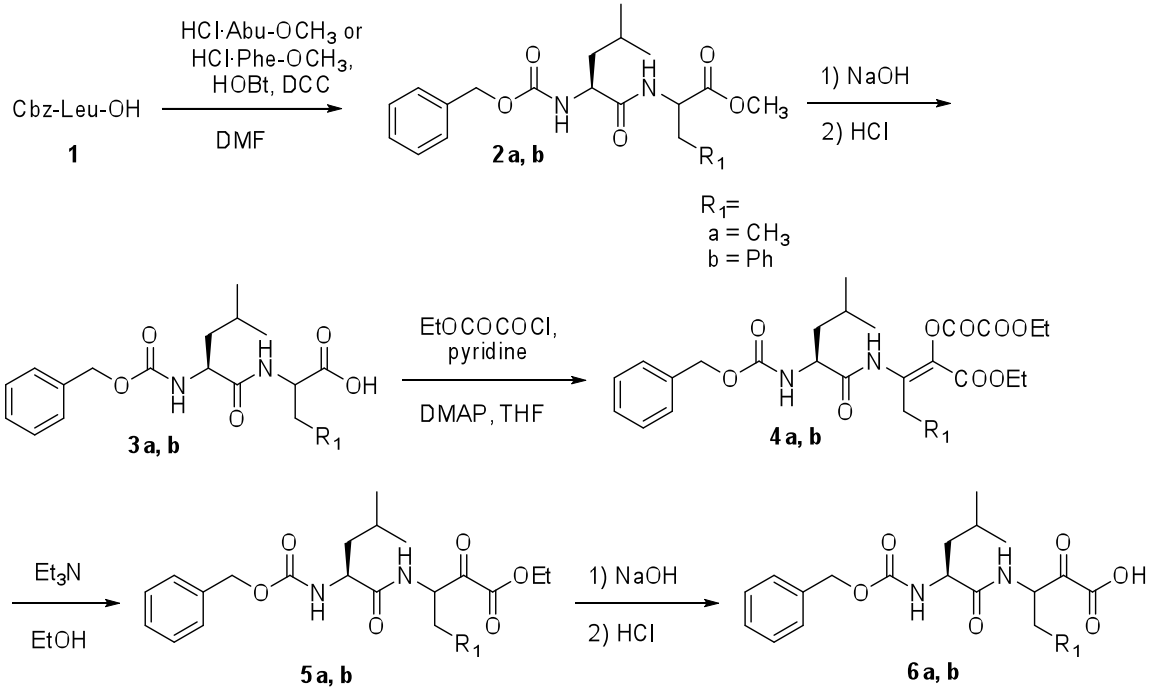


Figure 4.1.3. Synthesis of the Peptidyl α-Ketoacids.

Amines like 9-(3-aminopropyl)adenine (Figure 4.1.4) and 9-(3-aminopropyl)-2-methoxyadenine (Figure 4.1.5) were synthesized in three steps using the following

procedure.[151] Adenine and 2-methoxyadenine[152] were reacted with dihaloalkanes to add the linker by a single alkylation reaction. The halogen on the linker was then reacted with sodium azide to obtain the corresponding azide derivatives. The catalytic reduction of the azides in the presence of palladium activated on carbon and hydrogen gas gave the precursor amines.

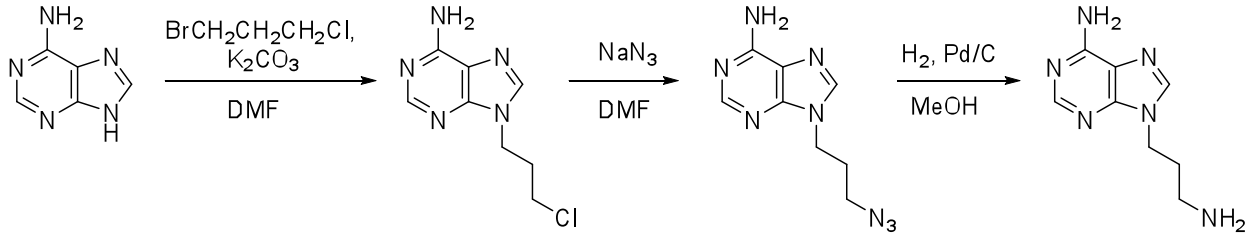
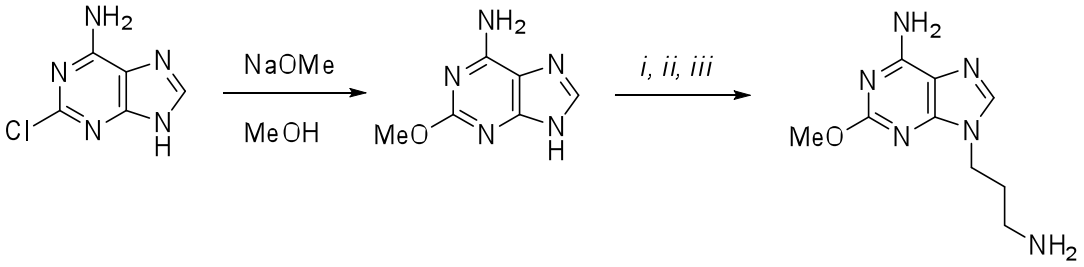


Figure 4.1.4. Synthesis of 9-(3-aminopropyl)adenine.



Reagents: (*i*) $BrCH_2CH_2CH_2Cl$, K_2CO_3, DMF (*ii*) NaN_3, DMF (*iii*) H_2, Pd/C, MeOH

Figure 4.1.5. Synthesis of 9-(3-aminopropyl)-2-methoxyadenine.

For the synthesis of 1-(3-aminopropyl)cytosine,[153] N-acetylcytosine was reacted with 1-bromo-3-chloropropane and then with sodium azide to form 1-(3-azidopropyl)-N-acetylcytosine. The acetyl group was deblocked with the ammonia and then the catalytic

reduction of azide was completed in the presence of palladium activated on carbon and hydrogen gas. (Figure 4.1.6)

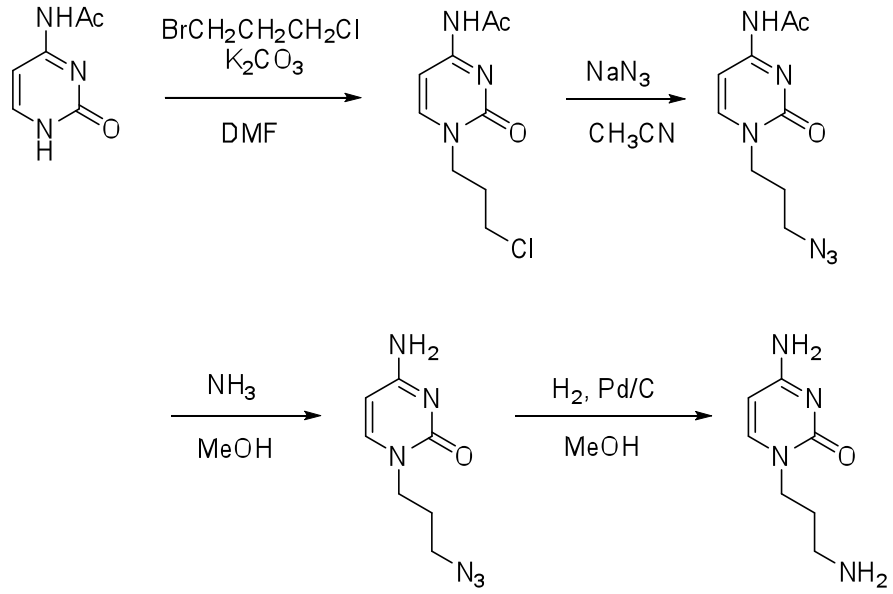
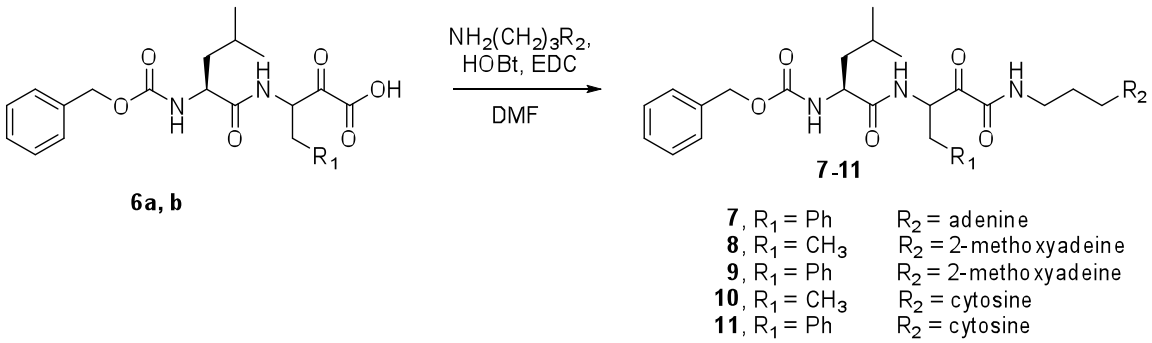


Figure 4.1.6. Synthesis of 1-(3-aminopropyl)cytosine.

The peptidyl α-ketoamides were obtained by using HOBt and EDC coupling of the corresponding α-ketoacid and the appropriate amine in low yields (Figure 4.1.7).



6a, b

7, R_1 = Ph R_2 = adenine
8, R_1 = CH_3 R_2 = 2-methoxyadeine
9, R_1 = Ph R_2 = 2-methoxyadeine
10, R_1 = CH_3 R_2 = cytosine
11, R_1 = Ph R_2 = cytosine

Figure 4.1.7. Synthesis of Peptidyl α-Ketoamides.

RESULTS AND DISCUSSION

A series of α-ketoamides as calpain inhibitors were synthesized and tested against calpain I, calpain II and cathepsin B (Table 4.1.1). The inhibition studies showed that the compounds with adenine and 2-methoxyadenine in the primed region were more potent than AK295 (Cbz-Leu-Abu-CONH-$(CH_2)_3$-morpholine) with a K_i value of 150 nM.

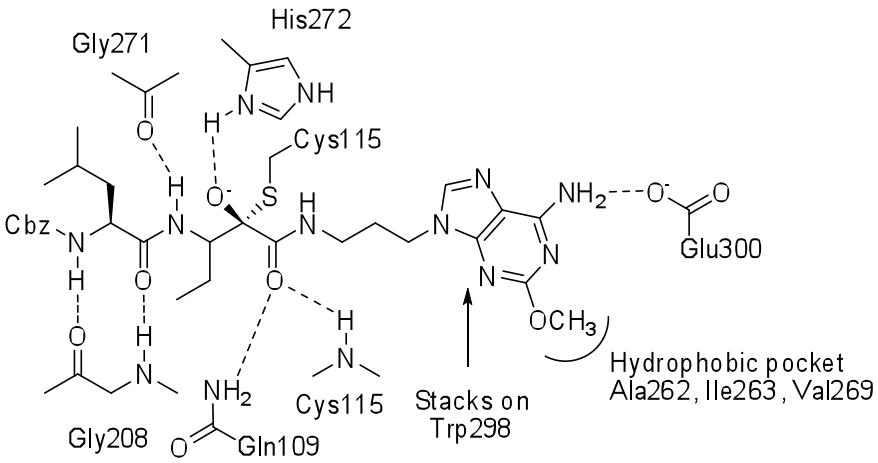


Figure 4.1.8. Proposed Binding Interactions of the Peptidyl α-Ketoamides with the Active Site of Calpain I.

The interactions of the peptidyl α-ketoamides with the active site of calpain I are shown in Figure 4.1.8. On the unprimed region, hydrogen bonds are formed between the inhibitor's peptide backbone atoms (amide nitrogen and carbonyl oxygen of the P2 residue and amide nitrogen of the P1 residue) and Gly208 and Gly271. The carbonyl of the carboxyamide provides stabilization via two hydrogen bonds with the side chain of Gln109 and the amide nitrogen of Cys115.

The best calpain I inhibitors were Cbz-Leu-Abu-CONH-$(CH_2)_3$-2-methoxyadenine (**8**) and Cbz-Leu-Phe-CONH-$(CH_2)_3$-2-methoxyadenine (**9**) with K_i values of 23 and 41 nM, respectively. Cbz-Leu-Abu-CONH-$(CH_2)_3$-adenine (ZLAK3001) and Cbz-Leu-Phe-CONH-$(CH_2)_3$-adenine (**7**) were slightly less potent than the 2-methoxyadenine derivatives but still have K_i values of 55 and 57 nM, respectively. The increased potency of 2-methoxyadenine derivatives confirmed that these compounds interact with the hydrophobic pocket in the primed region discovered in the crystal structure. There is a hydrogen bond between the amino group of the adenine and the side chain of Glu300, resulting in favorable electrostatic interaction. The peptidyl α-ketoamides containing adenine and 2-methoxyadenine are further stabilized by the aromatic stacking of the adenine ring on Trp298. Compounds **10** and **11** with cytosine in the primed side (Cbz-Leu-Abu-CONH-$(CH_2)_3$-cytosine, K_i = 165 nM; Cbz-Leu-Phe-CONH-$(CH_2)_3$-cytosine, K_i = 480 nM) were 3- to 8-fold less potent than the adenine derivatives. The amino group of the cytosine can also interact with the Glu300 and the lower potency of these compounds can be explained by the lack of aromatic stacking against Trp298. Among the compounds with nucleotide bases in the primed region, Abu in the P1 position is slightly favored over Phe.

Compounds containing N-methyl piperazine (ZLAK3002, K_i = 0.64 μM and ZLAK3006, K_i = 1.37 μM) and choline analogs (ZLAK3005, K_i = 26.5 μM; ZLAK3003, K_i = 0.226 μM; and ZLAK3004, K_i = 0.711 μM) in the primed side were less potent than AK295 and compounds with nucleotide bases in the primed side; but are still reasonable inhibitors of calpain I. The N-methyl piperazine derivatives ZLAK3002 and ZLAK3006 were less potent than the adenine derivatives ZLAK3001 and **7**, since these compounds

were lacking the stacking interactions with Trp298. Decreasing the alkyl spacer by one methylene group in compound ZLAK3004 resulted in 3-fold decrease in potency. Changing the amino acid in the P1 position from an Abu to a Phe resulted in 100-fold increase in potency in compound ZLAK3003.

Calpain II Inhibition. There is no crystal structure for calpain II but the active site of calpain II should be similar to the active of calpain I, therefore the same interactions shown in Figure 4.1.8 can occur between calpain II and peptidyl α-ketoamides. Cbz-Leu-Abu-CONH-$(CH_2)_3$-adenine (ZLAK3001) and Cbz-Leu-Phe-CONH-$(CH_2)_3$-adenine (**7**) were the most potent inhibitors of calpain II with K_i values of 70 nM and 68 nM, respectively. Introduction of the methoxy group to the C2 carbon of adenine did not change the potency much for Cbz-Leu-Abu-CONH-$(CH_2)_3$-2-methoxyadenine (**8**, K_i = 77 nM) but resulted in 3-fold decrease in potency for Cbz-Leu-Phe-CONH-$(CH_2)_3$-2-methoxyadenine (**9**, K_i = 209 nM). Cytosine derivatives Cbz-Leu-Abu-CONH-$(CH_2)_3$-cytosine (**10**, K_i = 1.14 μM) and Cbz-Leu-Phe-CONH-$(CH_2)_3$-cytosine (**11**, K_i = 0.438 μM) were less potent than the adenine and 2-methoxyadenine derivatives.

The N-methyl piperazine derivatives Cbz-Leu-Abu-CONH-$(CH_2)_3$-N-(1-methyl)piperazine (ZLAK3002, K_i = 0.286 μM) and Cbz-Leu-Abu-CONH-$(CH_2)_3$-N-(1-methyl)piperazine (ZLAK3006, K_i = 6.36 μM) were less potent than the adenine derivatives ZLAK3001 and **9**. The choline analogs ZLAK3005, ZLAK3003 and ZLAK3004 have K_i values of 25.9 μM, 0.844 μM and 3.52 μM; respectively. Again, decreasing the alkyl spacer length by one methylene group decreased the potency 4-fold for Cbz-Leu-Phe-CONH-$(CH_2)_2$-N-$(CH_3)_2$. Replacement of Abu with Phe in the P1

Table 4.1.1. Inhibition of Calpain I, Calpain II and Cathepsin B by Peptidiyl α-Ketoamides.

	Cal I	**Cal II**	**Cat B**
Compound	**K_i (μM)**	**K_i (μM)**	**K_i (μM)**
Cbz-Leu-Abu-CONH-$(CH_2)_3$-adenine	0.053	0.070	0.80
Cbz-Leu-Phe-CONH-$(CH_2)_3$-adenine	0.055	0.068	1.75
Cbz-Leu-Abu-CONH-$(CH_2)_3$-2-methoxyadenine	0.023	0.077	0.88
Cbz-Leu-Phe-CONH-$(CH_2)_3$-2-methoxyadenine	0.041	0.209	2.34
Cbz-Leu-Abu-CONH-$(CH_2)_3$-cytosine	0.165	1.14	0.75
Cbz-Leu-Phe-CONH-$(CH_2)_3$-cytosine	0.48	0.438	0.44
Cbz-Leu-Abu-CONH-$(CH_2)_3$-N-(1-methyl)piperazine	0.640	0.286	1.42
Cbz-Leu-Phe-CONH-$(CH_2)_3$-N-(1-methyl)piperazine	1.37	6.36	111
Cbz-Leu-Abu-CONH-$(CH_2)_3$-N-$(CH_3)_2$	26.5	25.9	44.9
Cbz-Leu-Phe-CONH-$(CH_2)_3$-N-$(CH_3)_2$	0.226	0.844	75.5
Cbz-Leu-Phe-CONH-$(CH_2)_2$-N-$(CH_3)_2$	0.711	3.52	475
Cbz-Leu-Abu-CONH-$(CH_2)_3$-morpholine	0.150	0.041	6.9

position resulted in 30-fold increase in potency for compound Cbz-Leu-Phe-CONH-$(CH_2)_3$-N-$(CH_3)_2$.

Selectivity. The heterocyclic peptidyl α-ketoamides displayed lower affinity toward cathepsin B, since they were designed to target calpains. Calpains cleave peptide substrates with a bulky residue (Phe, Abu or Met) in the P1 position and Val or Leu in the P2 position. Cathepsin B preferentially cleaves peptide substrates having a bulky residue such as Phe in the P2 position and a small hydrophobic residue in the P1 position. Cathepsin B was inhibited by peptidyl α-ketoamides but selectivity was obtained for calpain I and calpain II in various peptidyl α-ketoamides. The hydrophobic pocket found near the active site of calpain I was not observed in the primed side cleft of cathepsin B and addition of a hydrophobic group on the adenine ring resulted in an increased inhibitory potency toward calpain I and a decreased inhibitory potency toward cathepsin B. Cbz-Leu-Abu-CONH-$(CH_2)_3$-2-methoxyadenine with a K_i value of 23 nM is 3- and 38-fold poorer with calpain II and cathepsin B, respectively. Cbz-Leu-Phe-CONH-$(CH_2)_3$-2-methoxyadenine (K_i = 41 nM) is 5- and 57-fold more potent on calpain I than on calpain II and cathepsin B. Cbz-Leu-Phe-CONH-$(CH_2)_3$-cytosine was equally potent against calpains and cathepsin B. Cbz-Leu-Phe-CONH-$(CH_2)_3$-N-$(CH_3)_2$ having a K_i value of 0.226 μM is 3-, 334-fold more potent on calpain II and cathepsin B.

Mechanism of Inhibition. The mechanism of inhibition of calpain by α-ketoamides involves the formation of a reversible enzyme-inhibitor complex prior to the attack of the active site cysteine residue (Cys 115) on the keto carbonyl group of the α-ketoamides. This leads to the formation of a stable but reversible tetrahedral hemithioketal adduct (Figure 4.1.9). This hemithioketal adduct resembles the transition-

state for peptide bond hydrolysis, and it is quite stable. There is a hydrogen bond between the newly formed hydroxyl group and the imidazole ring of His272.

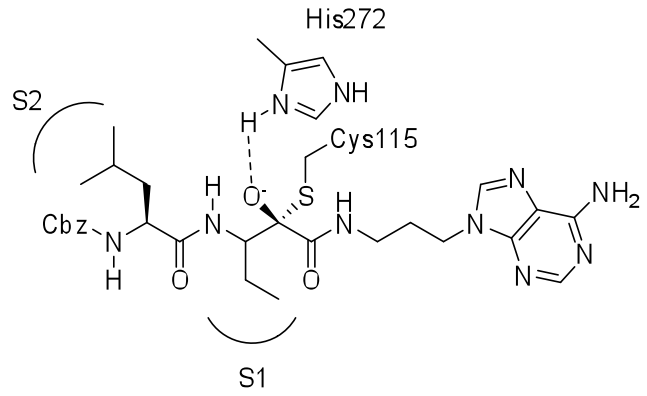


Figure 4.1.9. Mechanism of Interaction of Peptidyl α-Ketoamides with the Active Site of Calpain I.

CONCLUSIONS and SIGNIFICANCE

We have shown that peptidyl α-ketoamides with the general structure of Cbz-Leu-D,L-AA-CONH$(CH_2)_3$-R, where R is a heterocyclic base, are effective inhibitors of calpains. It has been observed that Abu, which is small hydrophobic residue, is slightly favored over the large hydrophobic residue Phe in the P1 position. We have obtained K_i values in the nanomolar range.

We have designed novel calpain inhibitors based on the crystal structure obtained with ZLAK3001. We have proposed to introduce a hydrophobic group on the adenine ring to to facilitate interactions with the hydrophobic pocket observed in the crystal

structure. Our design strategy worked and we successfully developed new calpain inhibitors with increased potency.

All these peptidyl α-ketoamides were designed based on the structure of AK295 which has been shown to be effective in the treatment of peripheral neuropathy. The problem with this compound was that it does not get to the brain. For calpain inhibitors to be used in the treatment of neurodegenerative diseases, they should be able to cross the BBB. Our aim was to synthesize effective calpain inhibitors that can cross the BBB. To do that, we have incorporated nucleoside and choline-like groups into the structure of our peptidyl α-ketoamides. There are specific transporters that can facilitate the transportation of nucleosides and choline to the brain.

AK295 in brain	0 microgram/g tissue
ZLAK3001 in brain	1.17 microgram/g tissue
ZLAK3002 in brain	1.16 microgram/g tissue

It has been shown that two of our compounds ZLAK3001 (with an adenine residue) and ZLAK3002 (with an N-methylpiperazine residue) have therapeutically useful concentrations in brain after administered to mice (Christina Hampton, Dr. Fernandez). We have synthesized peptidyl α-ketoamides with cytosine and 2-methoxyadenine in the primed side since these compounds also have the potential to cross the BBB.

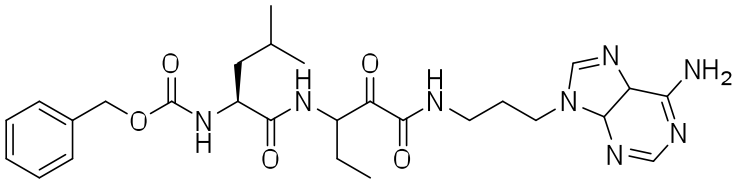
Increased levels of calpain activity have been observed in a number of neurodegenerative diseases including Alzheimer's, Huntington's and Parkinson's

diseases and multiple sclerosis. Development of selective calpain inhibitors that can cross the BBB is required for the treatment of these diseases. Here, we have shown that peptidyl α-ketoamides are novel calpain inhibitors that can cross or have the potential to cross the BBB. Heterocyclic peptidyl α-ketoamides have good potential to be used in the treatment of neurodegenerative diseases.

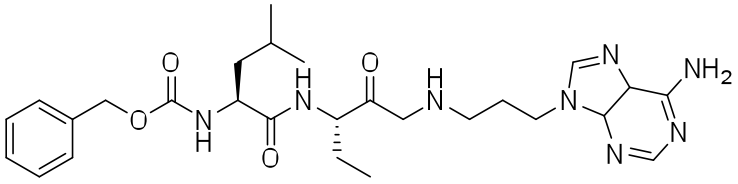
4.2. PEPTIDYL AMINOKETONES: A NEW CLASS OF INHIBITORS OF CALPAIN

INHIBITOR DESIGN

In an effort to find new reversible inhibitors of calpain, we have designed peptidyl aminoketones (Figure 4.2.1). These inhibitors yield a new patentable class of inhibitors that have the potential to cross the BBB. The design of peptidyl aminoketones were based on peptidyl α-ketoamides. The only difference between peptidyl aminoketones and peptidyl α-ketoamides was the carbonyl of the carboxyamide. We removed this carbonyl group to develop peptidyl aminoketones as a new class of inhibitors. The Cbz group was used as the N-terminal blocking group. Since the compounds possessing an Abu in the P1 position had a modest advantage over those with Phe for peptidyl α-ketoamides, we used Abu in the P1 position and Leu in the P2 position.



ZLAK3001



Peptidyl Aminoketone Derivative of ZLAK3001, **17**

Figure 4.2.1. Structures of ZLAK3001 and Peptidyl Aminoketone Derivative of ZLAK3001, **17**.

Peptidyl aminoketones have the advantage of being extended in the primed region of the inhibitor like the peptidyl α-ketoamides. We have incorporated nucleoside bases like adenine, 2-methoxyadenine and 2-chloroadenine to the primed side of the inhibitor to facilitate an aromatic stacking interaction between the adenine moiety and the side chain of Trp298. All these compounds can interact with Glu300 by forming a hydrogen bond with their amino groups. We have anticipated that 2-methoxyadenine and 2-chloroadenine derivatives would reach into the hydrophobic pocket formed by Ala262, Ile263 and Val269. We also hypothesized that the attachment of nucleoside bases to peptidyl aminoketones would result in new calpain inhibitor structures with improved transport across the BBB.

CHEMISTRY

The synthesis of peptidyl chloromethyl ketone was shown in Figure 4.2.2. Commercially available N-benzyloxycarbonyl leucine (**12**) was coupled to H•Abu-OCH_3 using HOBt and DCC coupling procedure to form the dipeptidyl methyl ester Cbz-Leu-Abu-OMe (**13**). The peptidyl methyl ester (**13**) was hydrolyzed with 1 M NaOH (1.1 eq) in methanol under standard deblocking conditions to obtain Cbz-Leu-Abu-OH (**14**). The dipeptidyl acid (**14**) was activated at the carboxy group by the mixed anhydride coupling method using isobutyl chloroformate (iBCF) and N-methyl morpholine (NMM) and reacted with ethereal diazomethane to form the corresponding peptidyl diazomethane ketone Cbz-Leu-Abu-CHN_2 (**15**). The diazomethane solution was prepared from *N*-methyl-*N*-nitroso-4-toluenesulfonamide according to the literature. The generated

peptidyl diazomethyl ketone (**15**) was reacted with HCl immediately to form the dipeptidyl chloromethyl ketone Cbz-Leu-Abu-CH_2Cl (**16**).

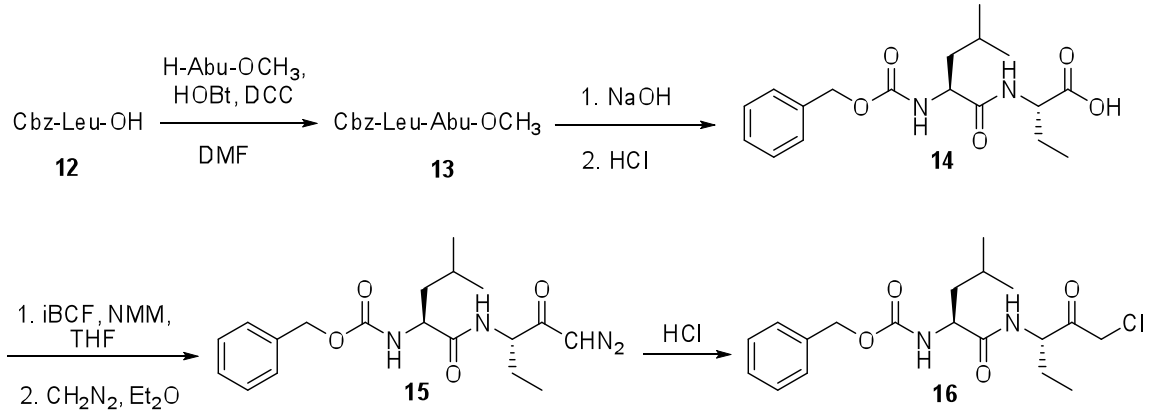


Figure 4.2.2. Synthesis of Dipeptidyl Chloromethyl Ketones.

The heterocyclic amine 9-(3-aminopropyl)-2-chloroadenine (Figure 4.2.3) was synthesized from 2-chloroadenine in three steps.[151] The heterocyclic base, 2-chloroadenine was reacted with 1-bromo-3-chloropropane to add the linker by a single alkylation reaction. The halogen on the linker was then reacted with sodium azide to obtain the corresponding azide derivative. The catalytic reduction of the azide in the presence of palladium activated on carbon and hydrogen gas gave the 9-(3-aminopropyl)-2-chloroadenine.

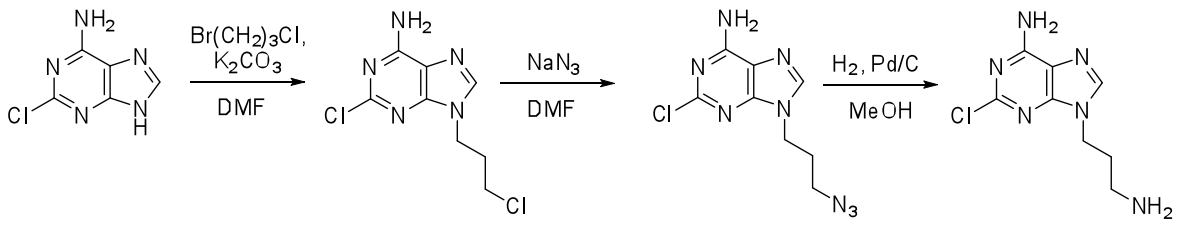


Figure 4.2.3. Synthesis of 9-(3-aminopropyl)-2-chloroadenine

Peptidyl chloromethyl ketone was dissolved in THF and then reacted with heterocyclic amines in the presence of triethylamine under refluxing conditions to form the peptidyl aminoketones (Figure 4.2.4).

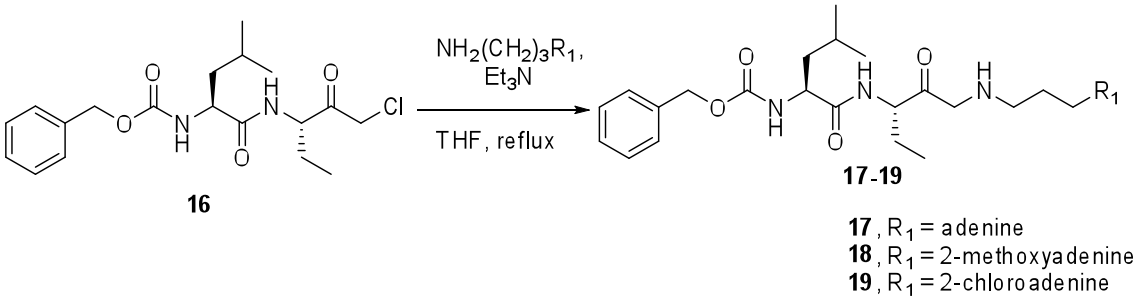


Figure 4.2.4. Synthesis of Peptidyl Aminoketones.

RESULTS AND DISCUSSION

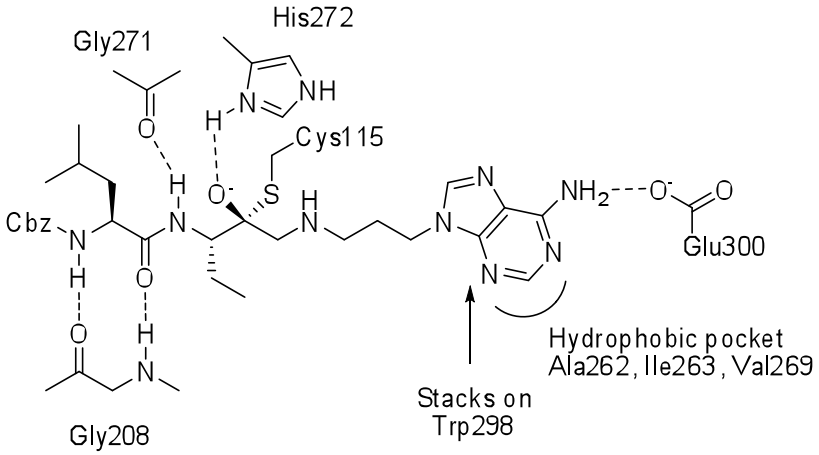


Figure 4.2.5. Proposed Binding Interactions of Peptidyl Aminoketones with the Active Site of Calpain I.

Table 4.2.1. Inhibition of Calpain I, Calpain II and Cathepsin B by Peptidyl Aminoketones.

	Compound	Cal I K_i (μM)	Cal II K_i (μM)	Cat B K_i (μM)	Cat B/ Cal I
17	Cbz-Leu-Abu-CH_2-NH-$(CH_2)_3$-adenine	5.59	0.785	4.57	0.82
18	Cbz-Leu-Abu-CH_2-NH-$(CH_2)_3$-2-methoxyadenine	3.50	231	7.69	2.20
19	Cbz-Leu-Abu-CH_2-NH-$(CH_2)_3$-2-chloroadenine	3.07	14.5	0.41	0.13

Calpain I Inhibition. Peptidyl aminoketones were less potent than peptidyl α-ketoamides, but they were still reasonable inhibitors of calpain I. The decreased potency of peptidyl aminoketones compared to peptidyl α-ketoamides can be explained by the disturbance of hydrogen bond network by removal of the carbonyl of the carboxyamide (Figure 4.2.5). Cbz-Leu-Abu-CH_2-NH-$(CH_2)_3$-adenine (**17**, K_i = 5.59 μM) were less potent than Cbz-Leu-Abu-CH_2-NH-$(CH_2)_3$-2-methoxyadenine (**18,** K_i = 3.50 μM) and Cbz-Leu-Abu-CH_2-NH-$(CH_2)_3$-2-chloroadenine (**19**, K_i = 3.07 μM). Introduction of hydrophobic groups to the C2 carbon of resulted in increased potency against calpain I where chlorine was favored over the methoxy group.

Calpain II Inhibition. Cbz-Leu-Abu-CH_2-NH-$(CH_2)_3$-adenine was the most potent inhibitor against calpain II with a K_i value of 0.785 μM. Replacement of adenine with 2-methoxyadenine in compound Cbz-Leu-Abu-CH_2-NH-$(CH_2)_3$-2-methoxyadenine (**18**, Ki = 231 μM) resulted in drastic decrease in potency. Cbz-Leu-Abu-CH_2-NH-

$(CH_2)_3$-2-chloroadenine with a K_i value of 14.5 μM was 20-fold less potent than Cbz-Leu-Abu-CH_2-NH-$(CH_2)_3$-adenine.

Cathepsin B Inhibition. Cbz-Leu-Abu-CH_2-NH-$(CH_2)_3$-2-chloroadenine was the most potent inhibitor of cathepsin B with a K_i value of 0.41 μM. Removal of the chlorine group in compound Cbz-Leu-Abu-CH_2-NH-$(CH_2)_3$-adenine (**17**, Ki = 4.57 μM) resulted in 10-fold decrease in potency. Introduction of methoxy group into the C2 carbon of adenine resulted in 2-fold decrease in compound Cbz-Leu-Abu-CH_2-NH-$(CH_2)_3$-2-methoxyadenine (**18**, K_i = 7.69 μM).

Selectivity. Cbz-Leu-Abu-CH_2-NH-$(CH_2)_3$-2-methoxyadenine with a K_i value of 3.50 μM toward calpain I was 66- and 2-fold poorer with calpain II and cathepsin B; respectively. Cbz-Leu-Abu-CH_2-NH-$(CH_2)_3$-adenine had a K_i value of 0.785 μM toward calpain II and this compound was 7-fold poorer with calpain I and almost equally potent toward cathepsin B with a K_i value of 4.57 μM. Cbz-Leu-Abu-CH_2-NH-$(CH_2)_3$-2-chloroadenine with a K_i value of 0.41 μM toward cathepsin B was 7- and 35-fold more potent on cathepsin B than on calpain I and calpain II, respectively.

CONCLUSION

Peptidyl aminoketones were designed as a new class of inhibitors for calpain with the general structure of Cbz-Leu-Abu-CH_2-NH-$(CH_2)_3$-R where the R group is adenine, 2-methoxyadenine and 2-chloroadenine. Peptidyl aminoketones were less potent than the peptidyl α-ketoamides. The carbonyl group of the carboxyamide was missing from the peptidyl aminoketones. The carbonyl oxygen of the carboxyamide was stabilized by two

hydrogen bonds from Gln109 and Cys115 in peptidyl α-ketoamides. Probably, the disturbance of this hydrogen network by removing the carbonyl group to form the peptidyl aminoketones resulted in less potent inhibitors.

Addition of hydrophobic groups to the adenine ring resulted in an increased inhibitory potency toward calpain I, where the chlorine group is favored over the methoxy group. On the other hand; introduction of hydrophobic groups led to a decrease in inhibitory potency toward calpain II. Peptidyl aminoketones inhibited calpains as well as cathepsin B effectively.

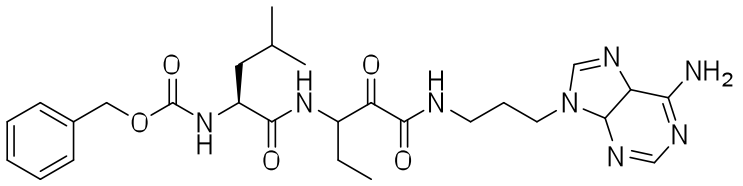
Calpains are involved in a wide range of biological processes and elevated levels of calpain activity have been observed in a number of neurodegenerative diseases. Calpains are ideal targets for the development of inhibitors. There are many isoforms of calpains and nonspecific inhibition of these isoforms can cause severe side effects. Therefore, reversible inhibitors of calpain are favored over the irreversible inhibitors. We have extended our inhibitors to the prime side by introducing nucleoside bases. These compounds have the potential to cross the BBB. Here, we report a new class of reversible calpain inhibitors with the potential to cross the BBB.

4.3. AZA-PEPTIDYL KETONES

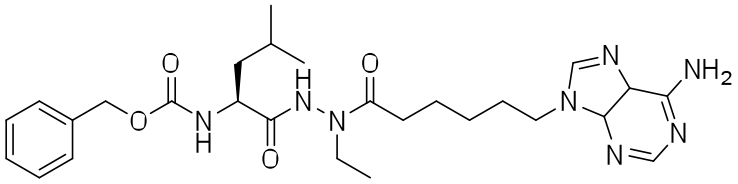
We have synthesized seven aza-peptide ketones, which are aza-derivatives of our peptidyl α-ketoamides.

INHIBITOR DESIGN

The design of aza-peptide ketone inihibitors was based on the peptidyl α-ketoamides synthesized by our group (Figure 4.3.1). In aza-peptide ketones, the α-carbon of the P1 amino acid residue (Abu) was replaced with a nitrogen atom to form the aza-amino acid residue (AAbu). This modification changes the geometry of the alpha position from tetrahedral to trigonal and reduces the electrophilicity of the P1 carbonyl group.[154] Some aza-peptides are more resistant to hydrolysis than their peptide analogs due to these reasons.[155] We used Leu in the P2 position and Cbz as the capping group same as α-ketomaides. To keep the bases in the primed side of the aza-peptide ketone inhibitors at the same distance as peptidyl α-ketomides we used an alkyl spacer. The carbonyl group that is attacked by the active site cysteine is in the same position as peptidyl α-ketomaides but the second carbonyl group and amide NH groups present in ketoamides were missing from the aza-peptide ketones. Aza-peptidyl ketone derivative of AK295, aza-peptidyl ketones with nucletiode bases (adenine, 2-chloroadenine, cytosine, etc) and choline type base (N-methyl piperazine) were synthesized.



ZLAK3001



Aza-peptidyl Ketone Derivative of ZLAK3001, **25**

Figure 4.3.1. Structures of ZLAK3001 and Aza-peptidyl Ketone Derivative of ZLAK3001, **25**.

CHEMISTRY

The synthesis of aza-peptidyl ketones were described in Figure 4.3.2. N-benzyloxycarbonyl leucyl hydrazide (**21**) was synthesized from the reaction of commercially available N-benzyloxycarbonyl leucine methyl ester (**20**) with excess hydrazine (10 eq) in methanol. For the introduction of the aminobutyric acid (Abu) side chain, the hydrazide Cbz-Leu-NH-NH_2 (**21**) was reacted with acetaldehyde in THF to form the hydrazone Cbz-Leu-NH-N=CH-CH_3 (**22**) and followed by subsequent reduction with $NaBH_3CN$ to form the hydrazide Cbz-Leu-NH-NH-CH_2CH_3 (**23**). The linker, 6-bromocaproic acid was coupled to the hydrazide (**23**) using HOBt and EDC coupling method to form Cbz-Leu-AAbu-$(CH_2)_5$-Br (**24)**. Cbz-Leu-AAbu-$(CH_2)_5$-Br was

dissolved in DMF and reacted with heterocyclic bases like, adenine, morpholine, etc. in the presence of K_2CO_3 to form the heterocyclic aza-peptidyl ketones (**25-31**).

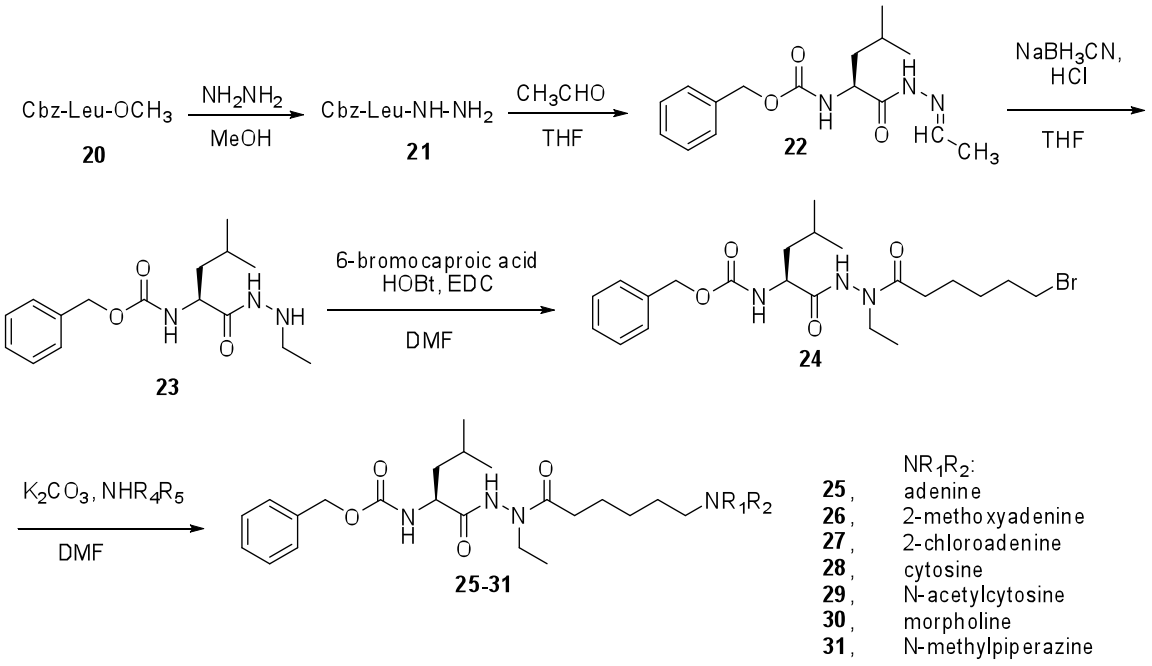


Figure 4.3.2. Synthesis of Aza-peptide Ketones.

RESULTS AND DISCUSSION

Calpain I Inhibition. Aza-peptide ketones did not inhibit calpain I probably due to the conversion of ketone carbonyl to an amide carbonyl. This modification resulted in a carbonyl which is much less susceptible to an attack by the active site cysteine for the formation of hemithioketal adduct. Previous work with E-64c analogs and aza-derivatives of these compounds showed that the change of the α-carbon to a nitrogen atom, to form the aza-residue, reduced the potency of aza-E-64c and derivatives for clan CA proteases (unpublished results). Modeling studies done by Dr. Juliana Asgian

showed that papain would not tolerate aza-E-64c. Another study with aza-peptide chloromethyl ketones discovered that changing the P1 residue from AGly to even a simple AAla residue resulted in complete loss of inhibitory activity against calpain.[154]

Table 4.3.1. Inhibition of Calpain I and Cathepsin B by Aza-peptide Ketones.

		Cal I	**Cat B**
	Compound	**K_i (mM)**	**K_i (mM)**
25	Cbz-Leu-AAbu-$(CH_2)_5$-adenine	N.I.[a]	N.I.[h]
26	Cbz-Leu-AAbu-$(CH_2)_5$-2-methoxyadenine	N.I.[b]	N.I.[i]
27	Cbz-Leu-AAbu-$(CH_2)_5$-2-chloroadenine	N.I.[c]	N.I.[j]
28	Cbz-Leu-AAbu-$(CH_2)_5$-N4-acetylcytosine	N.I.[d]	7.83
29	Cbz-Leu-AAbu-$(CH_2)_5$-cytosine	N.I.[e]	0.745
30	Cbz-Leu-AAbu-$(CH_2)_5$-morpholine	N.I.[f]	16.84
31	Cbz-Leu-AAbu-$(CH_2)_5$-N-Me-piperazine	N.I.[g]	N.I.[k]

[a] No inhibition at 1.2 μM, [b] No inhibition at 0.5 μM, [c] No inhibition at 1.2 μM, [d] No inhibition at 4.7 μM, [e] No inhibition at 2.4 μM, [f] No inhibition at 2.4 μM, [g] No inhibition at 2.4 μM, [h] 1% inhibition at 1.2 μM, [i] No inhibition at 2.4 μM, [j] 6% inhibition at 2.4 μM, [k] No inhibition at 2.4 μM.

Aza-peptides are more rigid than their peptide analogs due to their trigonal planar geometry and the inability of the N2-CO bond to rotate, as opposed to the α-CH-CO bond which can rotate. Clan CA proteases do not have the flexibility to accommodate the rigid aza-peptide compounds. The active site of papain and other clan CA proteases has a narrow, canyon like shape, and therefore clan CA proteases could not tolerate an aza-residue due to the topology of their active sites. Clan CD proteases, on the other hand, can tolerate aza-peptides very well since the active sites of clan CD proteases are wider and more solvent exposed than those of clan CA proteases. As a result, aza-peptide epoxides and Michael acceptors are potent inhibitors of clan CD proteases (Chapter 2).

Cathepsin B Inhibition. Cbz-Leu-AAbu-$(CH_2)_5$-adenine (**25**), Cbz-Leu-AAbu-$(CH_2)_5$-2-methoxyadenine (**26**) and Cbz-Leu-AAbu-$(CH_2)_5$-2-chloroadenine (**27**) did not inhibit cathepsin B. Cbz-Leu-AAbu-$(CH_2)_5$-N4-acetylcytosine (**28**) was a weak inhibitor of cathepsin B with a K_i value of 7.83 mM. Removal of the N-acetyl group resulted 10-fold increase in potency for compound Cbz-Leu-AAbu-$(CH_2)_5$-cytosine (**29**, K_i = 0.745 mM). Cbz-Leu-AAbu-$(CH_2)_5$-morpholine (**30**), aza-peptidyl ketone derivative of AK295, was a poor inhibitor of cathepsin B with a K_i value of 16. 84 mM. The aza-peptidyl ketone derivative of ZLAK3002, Cbz-Leu-AAbu-$(CH_2)_5$-N-Me-piperazine (**31**) did not inhibit cathepsin B.

CONCLUSION

We have designed aza-peptidyl ketones with the sturucture of Cbz-Leu-AAbu-$(CH_2)_5$-R as a new class of inhibitors for calpains. Design of these compounds was based on the peptidyl α-ketoamides. The differences between the aza-peptidyl ketones and the peptidyl α-ketoamides were the AAbu residue and the carboxyamide moiety. We have removed the carboxyamide moiety found in the peptidyl α-ketoamides and changed the α-carbon of Abu to nitrogen to form the aza-peptidyl ketones. When we tested these compounds with calpain, they did not inhibit the enzyme. Most of these compounds did not inhibit cathepsin B, either. Only a few of them inhibited cathepsin B and they were only poor inhibitors of the enzyme. The inability of these compounds to inhibit calpain and cathepsin B can be explained by the topologies of the active sites of these enzymes. Calpain and cathepsin B belong to clan CA of cysteine proteases. Clan CA cysteine proteaeses have a narrow active site and the active sites of this family of enzymes are not flexible enough to accommodate the rigid aza-peptides. We thought that the structural changes introduced by the replacement of α-carbon to nitrogen prevented the aza-peptidyl ketones to interact with the active site of the clan CA enzymes calpain and cathepsin B.

EXPERIMENTAL

Material and Methods. Materials were obtained from Acros, Bachem Bioscience Inc., or Sigma Aldrich and used without further purification. The purity of each compound was confirmed by TLC, ^{1}H NMR, MS, and elemental analysis.

Chemical shifts are reported in ppm relative to an internal standard (trimethylsilane). TLC was performed on Sorbent Technologies (250 μm) silica gel plates. The ^{1}H NMR spectra were obtained on a Varian Mercury 400 MHz spectrometer. Electrospray ionization (ESI), fast-atom-bombardment (FAB) and high-resolution mass spectrometry were obtained using Micromass Quattro LC and VG Analytical 70-SE instruments. Elemental analysis was carried out by Atlantic Microlab Inc., Norcross, GA.

Calpain I and Calpain II Assays. The fluorogenic substrate Suc-Leu-Tyr-AMC was obtained from Bachem. Calpain I from porcine erythrocytes and calpain II from porcine kidney were purchased from Calbiochem. Fluorescence was followed by Tecan Spectrafluor microplate reader. AMC was used as the calibration standard and calibration curves were plotted with RFU versus different concentrations of AMC within the range of 5-0.08 μM. Inhibitor stock solutions (50 mM to 0.01 mM) were prepared in DMSO and kept in the fridge prior to use. Calpain assays were performed in 50 mM Tris HCl, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% CHAPS, pH 7.5, 10 mM DTT, 5 mM $CaCl_2$ and three different substrate (Suc-Leu-Tyr-AMC) concentrations (0.8, 0.4, 0.2 μM as final concentrations in the assay). To this buffer (200 μL) was added a 10 μL aliquot of DMSO (control) or inhibitor solution in DMSO where the DMSO content of the final mixture did not exceed 5%. The reaction was initiated by the addition of a 2 μL aliquot of enzyme (with a final concentration of 10 nM) to the well. The reaction was monitored by the release of 7-amino-4-methylcoumarin (λ_{ex} = 360 nm, λ_{em} = 465 nm). Total volume in the reaction well was 212 μL and controls were run every hour. Velocities were determined at room temperature at five or more concentrations of inhibitor and at three fixed concentrations of substrate. Plot of 1/v versus [I] gave

intersecting lines with a correlation coefficient of $\geq$ 0.95. K_i values were determined by Dixon plots.[24]

Cathepsin B Assay. The fluorogenic substrate Cbz-Arg-Arg-AMC was obtained from Bachem. Cathepsin B from human liver was purchased from Calbiochem. Fluorescence was followed by Tecan Spectrafluor microplate reader. AMC was used as the calibration standard and calibration curve was plotted against RFU versus different concentrations of AMC within the range of 5-0.08 μM. Inhibitor stock solutions (50 mM to 0.01 mM) were prepared in DMSO and kept in the fridge prior to use. Cathepsin B assay was performed in 0.1 M NaHPO4, 1.25 mM EDTA, 0.01% Brij, pH 6.0 buffer and three different substrate (Cbz-Arg-Arg-AMC) concentrations (0.5, 0.2, 0.1 μM as final concentrations in the assay). To this buffer (200 μL) was added a 10 μL aliquot of DMSO (control) or inhibitor solution in DMSO where the DMSO content of the final mixture did not exceed 5%. The reaction was initiated by the addition of a 5 μL aliquot of activated enzyme (with a final concentration of 0.2 nM) to the well. The enzyme was activated by the addition of cathepsin B kinetic buffer (267 μL) and 0.1 M DTT (3 μL) to the enzyme stock solution (30 μL). The reaction was monitored by the release of 7-amino-4-methylcoumarin (λ_{ex} = 360 nm, λ_{em} = 465 nm). Total volume in the reaction well was 215 μL and controls were run every hour. Velocities were determined at room temperature at five or more concentrations of inhibitor and at three fixed concentrations of substrate. Plot of 1/v versus [I] gave intersecting lines with a correlation coefficient of $\geq$ 0.95. K_i values were determined by Dixon plots.[24]

Synthetic Procedures for the Synthesis of Peptidyl α-Ketoamides

General Procedure for the Synthesis of Dipeptidyl Methyl Ester. To a stirred solution of the Cbz-Leu-OH (1 eq) in DMF at -15 °C, HOBt (1.5 eq) was added. Hydrochloride salt of the amino acid methyl ester was pretreated with NMM (1.5 eq) at -15 °C DMF prior to addition. DCC (1.5 eq) was added to the solution and reaction mixture was allowed to react for 16 h at room temperature. The DMF was evaporated, and the residue was redissolved in EtOAc. The organic layer was washed with 2% citric acid, saturated $NaHCO_3$, saturated NaCl, dried over $MgSO_4$, and concentrated. Purification on a silica gel column with the proper eluent gave the product with yields of 68-76%.

Cbz-Leu-Abu-OMe (**2a**) was purified by column chromatography on silica gel using 9:1 EtOAc:hexane as the eluent; white solid, yield 68%. ^{1}H NMR ($CDCl_3$): 0.85-0.94 (m, 9H, 2 x Leu-CH_3 and Abu-CH_3), 1.48-1.91 (m, 5H, 2 x CH_2 and CH), 3.74 (s, 3H, OCH_3), 4.23 (m, 1H, α-H), 4.53 (m, 1H, α-H), 5.10 (s, 2H, CH_2), 5.26 (d, 1H, NH), 6.55 (d, 1H, NH), 7.31-7.37 (m, 5H, Ph). MS (ESI) *m/z* 365.2 [$(M + 1)^+$].

Cbz-Leu-Phe-OMe (**2b**) was purified by column chromatography on silica gel using 1:1 EtOAc:hexane as the eluent; white solid, yield 76%. ^{1}H NMR ($CDCl_3$): 0.90 (d, 6H, 2 x Leu-CH_3), 1.46 (m, 1H, CH), 1.62 (m, 2H, CH_2), 3.03-3.15 (m, 2H, CH_2), 3.70 (s, 3H, OCH_3), 4.19 (m, 1H, α-H), 4.84 (m, 1H, α-H), 5.05-5.12 (m, 2H, CH_2), 5.20 (d, 1H, NH), 7.08 (d, 1H, NH), 7.22-7.34 (m, 5H, Ph). MS (ESI) *m/z* 427.2 [$(M + 1)^+$].

General Procedure for the Synthesis of Dipeptidyl Acids. The dipeptidyl methyl esters were hydrolyzed in MeOH using 1 M aqueous NaOH (1.1 eq) under standard deblocking conditions.

Cbz-Leu-Abu-OH (**3a**), white solid, yield 89%. ^{1}H NMR (DMSO-d_6): 0.79-0.84 (m, 9H, 2 x Leu-CH_3 and Abu-CH_3), 1.41-1.73 (m, 5H, 2 x CH_2 and CH), 4.07-4.10 (m, 2H, 2 x α-H), 5.00 (s, 2H, CH_2), 7.32-7.40 (m, 6H, Ph and NH), 7.98-8.07 (d, 1H, NH). MS (ESI) *m/z* 351.1 [$(M + 1)^+$].

Cbz-Leu-Phe-OH (**3b**), white solid, yield 85%. ^{1}H NMR (DMSO-d_6): 0.87 (d, 6H, 2 x Leu-CH_3), 1.45-1.61 (m, 3H, CH_2 and CH), 2.96-3.20 (m, 2H, CH_2), 4.26 (m, 1H, α-H), 4.81 (m, 1H, α-H), 5.06-5.13 (m, 2H, CH_2), 5.54 (d, 1H, NH), 6.88 (d, 1H, NH), 7.10-7.34 (m, 5H, Ph). MS (ESI) *m/z* 413.1 [$(M + 1)^+$].

General Procedure for the Synthesis of Dipeptidyl α-Ketoesters. The dipeptide acid (1 eq) was dissolved in dry THF and 4-dimethylaminopyridine (0.05 eq), pyridine (3 eq), and ethyl oxalyl chloride (2.1 eq) were added sequentially. The resulting mixture was stirred at reflux temperature for 4 hours. After removing the heat source, 1 M HCl (50 mL) was added to the brown solution. The mixture was extracted with ethyl acetate (2 x 100 mL). The combined extract was washed with 100 mL of saturated NaCl, dried over $MgSO_4$ overnight, and filtered. Ethyl acetate was removed from the filtrate to give a mixture of products containing the dipeptidyl enol ester. The mixture of products was dissolved in 20 mL of absolute ethanol and stirred in an ice bath. Triethylamine (1 eq) was added in one portion and stirring was continued for 1 hour at room temperature. Solvent was removed from the final mixture using a rotary evaporator. The crude oil was subjected to column chromatography to give the dipeptidyl α-ketoester.

Cbz-Leu-Abu-COOEt (**5a**), light yellow oil, 76% yield. ^{1}H NMR ($CDCl_3$): 0.86-0.93 (m, 9H, 2 x Leu-CH_3 and Abu-CH_3), 1.26-1.37 (m, 4H, CH_3 and Leu-CH), 1.49-1.98 (m, 4H, Abu-CH_2, Leu-CH_2 and CH_3), 4.23-4.38 (m, 2H, 2 x α-H), 4.42-4.46 (m,

2H, CH_2), 5.03-5.13 (m, 2H, Cbz), 5.67-5.73 (m, 1H, NH), 7.21-7.32 (m, 6H, Ph and NH). HRMS (FAB) Calcd. for $C_{21}H_{31}N_2O_6$: 407.2182. Observed m/z 407.2178.

Cbz-Leu-Phe-COOEt (**5b**), light yellow oil, 68% yield. ^{1}H NMR ($CDCl_3$): 0.79-0.90 (m, 6H, 2 x Leu-CH_3), 1.22-1.61 (m, 6H, Leu-CH_2, CH_3 and Leu-CH), 2.93-3.07 (m, 1H, CH), 3.19-3.28 (m, 1H, CH), 4.14-4.33 (m, 4H, CH_2 and 2 x α-H), 5.08 (d, 2H, Cbz), 5.23-5.33 (m, 1H, NH), 6.77-6.84 (m, 1H, NH), 7.12-7.29 (m, 5H, Ph), 7.33 (s, 5H, Ph). HRMS (FAB) Calcd. for $C_{26}H_{33}N_2O_6$: 469.2339. Observed *m/z* 469.2337.

General Procedure for the Synthesis of Dipeptidyl α-Ketoacids. Dipeptidyl α-ketoesters (1eq) were dissolved in ethanol and 1 M NaOH solution (1.1 eq) was added in portions while stirring in an ice bath. The resulting mixture was stirred at room temperature for an hour and extracted with anhydrous ether (4 x 30 mL). The aqueous layer was acidified to pH 4 with 2 M HCl in an ice bath and extracted with Et_2O (2 x 50 mL). The combined ether extract was washed with saturated NaCl, dried over $MgSO_4$ overnight, and filtered. Ether was removed from the filtrate by evaporation and the product was dried under reduced pressure.

Cbz-Leu-Abu-COOH (**6a**), pale yellow hygroscopic flakes, 96% yield. ^{1}H NMR ($CDCl_3$): 0.91 (d, 9H, Abu-CH_3 and 2 x Leu-CH_3), 1.47-1.75 (m, 5H, Leu-CH_2, Abu-CH_2, Leu-CH), 4.13-4.35 (m, 2H, 2 x α-H), 5.04-5.13 (m, 3H, Cbz and NH), 7.32 (s, 5H, Ph), 8.35-8.41 (d, 1H, NH). HRMS (FAB) Calcd. for $C_{19}H_{27}N_2O_6$: 379.1869. Observed *m/z* 379.1870.

Cbz-Leu-Phe-COOH (**6b**), pale yellow hygroscopic flakes, 89% yield. ^{1}H NMR ($CDCl_3$): 0.77-0.86 (m, 6H, 2 x Leu-CH_3), 1.09-1.56 (m, 3H, Leu-CH_2 and Leu-CH), 2.49-2.51 (m, 1H, CH), 2.75-2.91 (m, 1H, CH), 4.01-4.08 (m, 2H, 2 x α-H), 4.89-5.06

(m, 3H, Cbz and NH), 7.18-7.40 (m, 10H, 2 x Ph), 8.49 (t, 1H, NH). HRMS (FAB) Calcd. for $C_{24}H_{29}N_2O_6$: 441.2026. Observed *m/z* 441.2025.

General Procedure for the Synthesis of 9-(3-aminopropyl)adenine. A mixture of adenine (1 eq), 1-bromo-3-chloropropane (4.3 eq), and potassium carbonate (2.35 eq) in DMF (200 mL) was stirred at room temperature under argon for 4 days, filtrated, and evaporated to dryness. The crude product was washed with water and dried. Recrystallization from ethanol gave 9-(3-chloropropyl)adenine in 59% yield. MS (ESI) *m/z* 212.0 $[(M + 1)^+]$.

A mixture of 9-(3-chloropropyl)adenine (1 eq) and sodium azide (3 eq) in DMF was stirred at 80 °C for 24 hours, cooled to room temperature, and filtered. The solid was washed with CH_2Cl_2. The solvent was removed from the combined filtrates and the residue was taken up in water with sonication. The aqueous layer was extracted with CH_2Cl_2 (3 x 60 mL). After removing solvent, the crude product was recrystallized from ethanol to give 9-(3-azidopropyl)adenine as a white crystalline solid in 81% yield. ^{1}H NMR (DMSO-d_6): 2.04 (m, 2H, CH_2), 3.36 (m, 2H, CH_2), 4.19 (m, 2H, CH_2), 7.22 (s, 2H, NH_2), 8.12 (s, 2H, CH of adenine).

A mixture of 9-(3-azidopropyl)adenine and 5 % palladium on carbon in methanol was reacted with hydrogen gas at room temperature for 22 hours. The catalyst was removed by filtration, the solvent removed to give 9-(3-aminopropyl)adenine as a white solid in 76% yield. ^{1}H NMR (DMSO-d_6): 1.80 (m, 2H, CH_2), 2.45 (m, 2H, CH_2), 3.35 (s, 2H, NH_2), 4.20 (m, 2H, CH_2), 7.20 (s, 2H, NH_2), 8.10 (s, 2H, CH). MS (ESI) *m/z* 193.0 $[(M + 1)^+]$.

General Proceure for the Synthesis of 9-(3-aminopropyl)-2-methoxyadenine. A mixture of 2-chloroadenine (1 eq), sodium methoxide (7.5 eq) in anhydrous methanol (50 mL) was sealed in pressure vessel. The reaction mixture was heated to an internal temperature of 100 °C and the reaction mixture was maintained at 100 °C for 24 hours before cooling to room temperature. Once cooled, the pressure vessel was opened and the suspension was diluted with water (50 mL). The resulting solution was evaporated under reduced pressure to give a final volume of 70 mL; water (30 mL) was added to this solution to give a final volume of 100 mL. The solution was transferred to a 3-neck flask equipped with a stirrer, thermometer and pH meter. The solution was heated to 60°C (internal temperature) and 50% aq. HCl was added to adjust the pH to 9.5. The resulting suspension was stirred at 60 °C for 1 hour and cooled slowly to room temperature and stirred for 16 hours. The suspension was filtered and the filter cake was washed with water (10 mL) and methanol (2 x 10 mL). The solid was dried under vacuum to give 2-methoxyadenine in 70% yield. ^{1}H NMR (DMSO-d_6): 3.76 (s, 3H, OCH_3), 7.12 (s, 2H, NH_2), 7.86 (s, 1H, CH).

A mixture of 2-methoxyadenine (1 eq), 1-bromo-3-chloropropane (4.3 eq), and potassium carbonate (2.35) in DMF (200 mL) was stirred at room temperature under argon for 4 days, filtered, and evaporated to dryness. The crude product was purified by column chromatography and gave 9-(3-chloropropyl)-2-methoxyadenine in 66% yield. MS (ESI) *m/z* 241.9 [$(M + 1)^+$].

A mixture of 9-(3-chloropropyl)-2-methoxyadenine (1 eq) and sodium azide (3 eq) in DMF was stirred at 80 °C for 24 hours, cooled to room temperature, and filtered. The crude product was purified by column chromatography to give 9-(3-azidopropyl)-2-

methoxyadenine as a white crystalline solid in 74% yield. ^{1}H NMR (DMSO-d_6): 1.99-2.06 (m, 2H, CH_2), 3.33-3.37 (m, 2H, CH_2), 3.80 (s, 3H, OCH_3), 4.10 (t, 2H, CH_2), 7.21 (s, 2H, NH_2), 7.92 (s, 1H, CH). MS (ESI) *m/z* 249.0 [$(M + 1)^+$].

A mixture of 9-(3-azidopropyl)-2-methoxyadenine and 5 % palladium on carbon in methanol was reacted with hydrogen gas at room temperature for 20 hours. The catalyst was removed by filtration, the solvent removed to give 9-(3-aminopropyl)-2-methoxyadenine as a white solid in 75% yield. ^{1}H NMR (DMSO-d_6): 1.82-1.85 (m, 2H, CH_2), 2.46-2.49 (m, 2H, CH_2), 3.03 (s, 2H, NH_2), 3.79 (s, 3H, OCH_3), 4.09 (t, 2H, CH_2), 7.20 (s, 2H, NH_2), 7.92 (s, 1H, CH). MS (ESI) *m/z* 223.2 [$(M + 1)^+$].

General Procedure for the Synthesis of 1-(3-aminopropyl)cytosine. A mixture of N4-acetylcytosine (1eq), 1-bromo-3-chloropropane (1.5 eq), and potassium carbonate (1.5 eq) in DMF was stirred at room temperature under argon for 4 days, filtered and evaporated to dryness. The crude product was purified by column chromatography and gave 1-(3-chloropropyl)-N4-acetylcytosine. MS (ESI) *m/z* 230.0 [$(M + 1)^+$].

A mixture of 1-(3-chloropropyl)-N4-acetylcytosine and sodium azide in acetonitrile was refluxed for 24 hours, cooled to room temperature, and filtered. The crude product was purified by silica gel column chromatography and gave 1-(3-azidopropyl)-N4-acetylcytosine as a white solid. ^{1}H NMR (DMSO-d_6): 1.85-1.91 (m, 2H, CH_2), 2.06 (s, 3H, CH_3), 3.35-3.39 (t, 2H, CH_2), 3.81-3.84 (t, 2H, CH_2), 7.12 (d, 1H, CH), 8.04 (s, 1H, CH), 10.79 (s, 1H, NH). MS (ESI) *m/z* 237.0 [$(M + 1)^+$].

A mixture of 1-(3-azidopropyl)-N4-acetylcytosine was reacted with 7N ammonia solution in methanol at room temperature for 2 days to give 1-(3-azidopropyl)cytosine as a white solid. ^{1}H NMR (DMSO-d_6): 1.73-1.83 (m, 2H, CH_2), 3.31-3.34 (t, 2H, CH_2),

3.64-3.67 (t, 2H, CH_2), 5.62 (d, 1H, CH), 7.01 (d, 2H, NH_2), 7.53 (s, 1H, CH). MS (ESI) *m/z* 195.0 [$(M + 1)^+$].

A mixture of 1-(3-azidopropyl)cytosine and 5% palladium on carbon in methanol was reacted with hydrogen gas at room temperature for 8 h. The catalyst was removed by filtration, the solvent was removed to give 1-(3-aminopropyl)cytosine as a white solid. ^{1}H NMR (DMSO-d_6): 1.66 (m, 2H, CH_2), 2.96 (t, 2H, CH_2), 2.96 (s, 2H, CH_2), 3.57-3.67 (m, 4H, CH_2 and NH_2), 5.59 (d, 1H, CH), 6.97 (d, 2H, NH_2), 7.54 (d, 1H, CH). MS (ESI) *m/z* 169.9 [$(M + 1)^+$].

General Procedure for the Synthesis of Peptidyl α-Ketoamides by the HOBt and EDC Coupling Method. To a stirred solution of the dipeptidyl α-ketoacid (1.5 eq) in DMF at -10 °C was added HOBt (1.5 eq), the heterocyclic amine (1 eq) and EDC (1.5 eq) was added. The mixture was allowed to react for 16 h at room temperature. The DMF was evaporated, and the residue was redissolved in EtOAc. The organic layer was washed with 2% citric acid, saturated $NaHCO_3$, saturated NaCl, dried over $MgSO_4$, and concentrated. Column chromatography on silica gel afforded the peptidyl α-ketoamides.

(7, Cbz-Leu-Phe-CONH-$(CH_2)_3$-adenin-9-yl). The ketoamide product Cbz-Leu-Phe-CONH-$(CH_2)_3$-adenin-9-yl was obtained from 9-(3-aminopropyl)adenine and the ketoacid Cbz-Leu-Phe-COOH using the EDC and HOBt coupling method, purified by column chromatography on silica gel with 85:15 CH_2Cl_2:MeOH as the eluent, then recrystallization from EtOAc/hexane to give a white solid (21% yield). ^{1}H NMR (DMSO-d_6): 0.69-0.86 (m, 9H, 2 x Leu-CH_3 and Abu-CH_3), 1.15-1.36 (m, 5H, 2 x CH_2 and CH), 1.92-2.00 (m, 2H, CH_2), 3.04-3.15 (m, 4H, 2 x CH_2), 4.07-4.14 (m, 2H, CH_2 and 2 x α-H), 4.95-5.01 (m, 2H, Cbz), 5.18 (s, 1H, NH), 7.12-7.40 (m, 12H, 2 x Ph and

NH_2), 8.08-8.14 (m, 2H, 2 x Adenine-CH), 8.33-8.39 (d, 1H, NH), 8.83-8.89 (t, 1H, NH). HRMS (FAB) Calcd. for $C_{32}H_{39}N_8O_5$: 615.3043. Observed *m/z* 615.3094. Anal. Calcd. for $C_{32}H_{38}N_8O_5 \cdot 0.75H_2O$: C, 61.18; H, 6.34; N, 17.84. Found: C, 61.14; H, 6.37; N, 17.89.

3-(Benzyloxycarbonyl-L-leucylamino)-N-(3-(6-amino-2-methoxy-9H-purin-9-yl)propyl)-2-oxopentanamide (8, Cbz-Leu-Abu-CONH-$(CH_2)_3$-2-methoxyadenin-9-yl). The ketoamide product Cbz-Leu-Abu-CONH-$(CH_2)_3$-2-methoxyadenin-9-yl was obtained from of 9-(3-aminopropyl)-2-methoxyadenine and the ketoacid Cbz-Leu-Abu-COOH using the EDC and HOBt coupling method, purified by column chromatography on silica gel with 85:15 CH_2Cl_2:MeOH as the eluent, then recrystallization from EtOAc/hexane to give a yellowish white solid (26% yield). ^{1}H NMR (DMSO-d_6): 0.72-0.93 (m, 9H, 2 x CH_3 of Leu and CH_3 of Abu), 1.37-1.60 (m, 4H, CH_2 of Leu and CH_2 of Abu), 1.76 (m, 1H, CH of Leu), 1.93-1.97 (m, 2H, CH_2), 3.09 (m, 2H, CH_2), 3.78 (s, 3H, OCH_3), 3.98-4.14 (m, 3H, CH_2 and α-H), 4.84 (m, 1H, α-H), 4.99 (s, 2H, Cbz), 7.20 (s, 2H, NH_2), 7.28-7.40 (m, 6H, Ph and NH), 7.93 (s, 1H, CH of adenine), 8.24-8.31 (m, 1H, NH), 8.77 (m, 1H, NH). HRMS (FAB) Calcd. for $C_{28}H_{41}N_6O_6$: 583.2993. Observed *m/z* 583.2900. Anal. Calcd. for $C_{28}H_{39}N_8O_6 \cdot 0.55H_2O$: C, 56.75; H, 6.65; N, 18.91. Found: C, 56.72; H, 6.63; N, 18.97.

(9, Cbz-Leu-Phe-CONH-$(CH_2)_3$-2-methoxyadenin-9-yl). The ketoamide product Cbz-Leu-Phe-CONH-$(CH_2)_3$-2-methoxyadenin-9-yl was obtained from of 9-(3-aminopropyl)-2-methoxyadenine and the ketoacid Cbz-Leu-Phe-COOH using the EDC and HOBt coupling method, purified by column chromatography on silica gel with 85:15 CH_2Cl_2:MeOH as the eluent, then recrystallization from EtOAc/hexane to give a yellow

solid (24% yield). ^{1}H NMR (DMSO-d_6): 0.73-0.75 (d, 6H, 2 x CH_3 of Leu), 1.11-1.35 (m, 4H, CH_2 Leu and CH_2 of Phe), 1.54 (m, 1H CH of Leu), 1.95-1.98 (m, 2H, CH_2), 3.09-3.12 (m, 2H, CH_2), 3.77 (s, 3H, OCH_3), 4.03 (m, 3H, CH_2 and α-H), 4.97 (s, 2H, Cbz), 5.17 (m, 1H, α-H), 7.20-7.33 (m, 12H, 2 x Ph and NH_2), 7.93 (d, 1H, CH of adenine), 8.38 (d, 1H, NH), 8.84 (m, 1H, NH). HRMS (FAB) Calcd. for $C_{33}H_{41}N_6O_6$: 645.3149. Observed *m/z* 645.3067. Anal. Calcd. for $C_{33}H_{40}N_8O_6 \cdot 0.25H_2O$: C, 61.05; H, 6.29; N, 17.26. Found: C, 59.98; H, 6.22; N, 17.37.

(10, Cbz-Leu-Abu-CONH-$(CH_2)_3$-cytosin-3-yl). The ketoamide product Cbz-Leu-Abu-CONH-$(CH_2)_3$-cytosin-3-yl was obtained from of 1-(3-aminopropyl)cytosine and the ketoacid Cbz-Leu-Abu-COOH using the EDC/HOBt coupling method, purified by column chromatography on silica gel with 85:15 CH_2Cl_2:MeOH as the eluent, then recrystallization from EtOAC/hexane to give a yellowish white solid (11% yield). ^{1}H NMR (DMSO-d_6): 0.72-0.95 (m, 9H, 2 x CH_3 of Leu and CH_3 of Abu), 1.41-1.73 (m, 5H, CH_2 of Leu, CH_2 of Abu and CH of Leu), 2.13-2.19 (m, 2H, CH_2), 3.07 (m, 2H, CH_2), 3.56 (m, 1H, α-H), 3.93-4.10 (m, 3H, α-H and CH_2), 4.99 (s, 2H, Cbz), 5.60 (d, 1H, CH of cytosine), 6.97 (d, 2H, NH_2), 7.32-7.44 (m, 6H, Ph and NH), 7.68 (d, 1H, CH of cytosine), 8.22-8.28 (m, 1H, NH), 8.73 (m, 1H, NH). HRMS (FAB) Calcd. for $C_{26}H_{37}N_6O_6$: 529.2775. Observed *m/z* 529.2781. Anal. Calcd. for $C_{26}H_{36}N_6O_6 \cdot 1$EtOAc: C, 58.43; H, 7.19; N, 13.63. Found: C, 58.37; H, 7.38; N, 13.62.

(11, Cbz-Leu-Phe-CONH-$(CH_2)_3$-cytosin-3-yl). The ketoamide product Cbz-Leu-Phe-CONH-$(CH_2)_3$-cytosin-3-yl was obtained from of 1-(3-aminopropyl)cytosine and the ketoacid Cbz-Leu-Phe-COOH using the EDC and HOBt coupling method, purified by column chromatography on silica gel with 85:15 CH_2Cl_2:MeOH as the eluent,

then recrystallization from EtOAc/hexane to give a yellow solid (12% yield). ^{1}H NMR (DMSO-d_6): 0.74-0.76 (d, 6H, 2 x CH_3 of Leu), 1.11-1.37 (m, 4H, CH_2 Leu and CH_2 of Phe), 1.59 (m, 1H CH of Leu), 2.11-2.15 (m, 2H, CH_2), 3.01-3.10 (m, 2H, CH_2), 3.82-4.03 (m, 3H, α-H and CH_2), 4.99 (s, 2H, Cbz), 5.18 (m, 1H, NH), 5.61 (d, 1H, CH of cytosine), 6.97 (d, 2H, NH_2), 7.13-7.57 (m, 11H, 2 x Ph and CH of cytosine), 8.36 (d, 1H, NH), 8.80 (m, 1H, NH). HRMS (FAB) Calcd. for $C_{31}H_{39}N_6O_6$: 591.2886. Observed *m/z* 591.2852. Anal. Calcd. for $C_{31}H_{38}N_6O_6 \cdot 1$EtOAc: C, 61.93; H, 6.83; N, 12.38. Found: C, 61.71; H, 6.82; N, 12.27.

Synthetic Procedures for the Synthesis of Peptidyl AminoKetones

General Procedure for the Synthesis of Dipeptidyl Methyl Ester (13, Cbz-Leu-(L)Abu-OMe). First, commercilally available L-(2)-aminobutyric acid is esterified with $SOCl_2$ in the presence of MeOH and then coupled to Cbz-Leu-OH using HOBt/DCC coupling method. To a stirred solution of the Cbz-Leu-OH (1 eq) in DMF at -15 °C, HOBt (1.5 eq) was added. Hydrochloride salt of the L-Abu-OMe was pretreated with NMM (1.5 eq) at -15 °C DMF prior to addition. DCC (1.5 eq) was added to the solution and reaction mixture was allowed to react for 16 h at room temperature. The DMF was evaporated, and the residue was redissolved in EtOAc. The organic layer was washed with 2% citric acid, saturated $NaHCO_3$, saturated NaCl, dried over $MgSO_4$, and concentrated. Purification on a silica gel column with the proper eluent gave the product with 65% yield. ^{1}H NMR ($CDCl_3$-d): 0.82-0.87 (m, 9H, 2 x Leu-CH_3 and Abu-CH_3), 1.41-1.74 (m, 5H, 2 x CH_2 and CH), 3.59 (s, 3H, OCH_3), 4.06-4.18 (m, 2H, 2 x α-H), 5.00 (s, 2H, CH_2), 7.29-7.39 (m, 6H, Ph and NH), 8.19 (d, 1H, NH).

General Procedure for the Synthesis of Dipeptidyl Acid (14, Cbz-Leu-(L)Abu-OH). Cbz-Leu-(L)Abu-OH was obtained by hydrolysis of Cbz-Leu-(L)Abu-OMe in MeOH using NaOH (1 M aqueous, 1.1 eq); white solid, yield 88%. ^{1}H NMR (DMSO-d_6): 0.79-0.84 (m, 9H, 2 x Leu-CH_3 and Abu-CH_3), 1.41-1.73 (m, 5H, 2 x CH_2 and CH), 4.07-4.10 (m, 2H, 2 x α-H), 5.00 (s, 2H, CH_2), 7.32-7.40 (m, 6H, Ph and NH), 7.98-8.07 (d, 1H, NH).

General Procedure for the Synthesis of Dipeptidyl Chloromethyl Ketone (16, Cbz-Leu-Abu-CH_2Cl). To a cooled solution of Cbz-Leu-Abu-OH (1 eq) and NMM (1 eq) in dry THF (50 mL) was added iBCF (0.9 eq) at -10 °C with stirring. After 15 min, the mixture was filtered rapidly and the filtrate was added dropwise to cold CH_2N_2 in ether with stirring. When the addition was complete, the reaction mixture was left at room temperature for 1 h. The reaction mixture was cooled down to 5 °C and 4 M HCl in dioxane was added dropwise until the mixture was colorless. The solvents were removed under reduced pressure and the crude product was purified by column chromatography using 5%MeOH/CH_2Cl_2 as eluent to give a white solid with 50% yield. ^{1}H NMR (DMSO-d_6): 0.81-0.88 (m, 9H, 2 x Leu-CH_3 and Abu-CH_3), 1.39-1.77 (m, 5H, 2 x CH_2 and CH), 4.04 (m, 1H, α-H), 4.24 (m, 1H, α-H), 4.54 (s, 2H, CH_2), 5.01 (s, 2H, CH_2), 7.33-7.48 (m, 6H, Ph and NH), 8.35 (d, 1H, NH). MS (ESI) *m/z* 383.2.0 [(M + 1)$^+$].

General Procedure for the Synthesis of 9-(3-aminopropyl)-2-chloroadenine. A mixture of 2-chloroadenine (1 eq), 1-bromo-3-chloropropane (4.3 eq), and potassium carbonate (2.35 eq) in DMF (200 mL) was stirred at room temperature under argon for 4 days, filtered, and evaporated to dryness. The crude product was purified by column

chromatography and gave 9-(3-chloropropyl)-2-chloroadenine in 57% yield. ^{1}H NMR (DMSO-d_6): 2.23-2.27 (m, 2H, $NCH_2CH_2CH_2Cl$), 3.63 (t, 2H, $NCH_2CH_2CH_2Cl$), 4.21 (t, 2H, $NCH_2CH_2CH_2Cl$), 7.76 (s, 2H, NH_2), 8.14 (s, 1H, Adenine). MS (ESI) *m/z* 245.9 $[(M + 1)^+]$.

A mixture of 9-(3-chloropropyl)-2-chloroadenine (1 eq) and sodium azide (3 eq) in DMF was stirred at 80 °C for 24 hours, cooled to room temperature, and filtered. The crude product was purified by column chromatography to give 9-(3-azidopropyl)-2-chloroadenine as a white crystalline solid in 34% yield. ^{1}H NMR (DMSO-d_6): 1.98-2.05 (m, 2H, $NCH_2CH_2CH_2N_3$), 3.34-3.39 (m, 2H, $NCH_2CH_2CH_2N_3$), 4.15 (t, 2H, $NCH_2CH_2CH_2N_3$), 7.75 (s, 2H, NH_2), 8.14 (s, 1H, Adenine). MS (ESI) *m/z* 253.0 $[(M + 1)^+]$.

A mixture of 9-(3-azidopropyl)-2-chloroadenine and 5 % palladium on carbon in methanol was reacted with hydrogen gas at room temperature for 20 hours. The catalyst was removed by filtration, the solvent removed to give 9-(3-aminopropyl)-2-chloroadenine as a white solid in 87% yield. ^{1}H NMR (DMSO-d_6): 2.09-2.13 (m, 2H, CH_2), 2.74 (m, 2H, CH_2), 3.37 (s, 2H, NH_2), 4.22-4.27 (m, 2H, CH_2), 7.26 (s, 1H, CH), 8.20 (s, 2H, NH_2).

General Procedure for the Synthesis of Peptidyl AminoKetones. A solution of base (1 eq), Cbz-Leu-Abu-CH_2Cl (1.1 eq) and Et_3N (2 eq) in dry THF was heated at reflux for 24 h. The crude product was purified by column chromatography to give the aminoketones.

(17, Cbz-Leu-Abu-CH_2-NH-$(CH_2)_3$-adenine). This compound was obtained by the reaction of Cbz-Leu-Abu-CH_2Cl and 9-(3-aminopropyl)adenine. The crude product

was purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (9% yield). ^{1}H NMR (DMSO-d_6): 0.54-0.59 (m, 3H, Abu-CH_3), 0.79-0.88 (m, 6H, 2 x Leu-CH_3), 1.31-1.75 (m, 5H, 2 x CH_2 and CH), 1.87-2.26 (m, 6H, 3 x CH_2), 2.62 (t, 1H, NH), 3.97-4.15 (m, 4H, 2 x α-H and CH_2), 4.95-5.07 (m, 2H, CH_2), 7.15-7.30 (m, 7H, Ph and NH_2), 7.58 (d, 1H, NH), 8.02-8.07 (m, 3H, 2 x Adenine-CH and NH). HRMS (FAB) Calcd. for $C_{27}H_{39}N_8O_4$: 539.3094. Observed *m/z* 539.3088. Anal. Calcd. for $C_{27}H_{38}N_8O_4 \cdot 0.1H_2O$: C, 60.00; H, 7.12; N, 20.73. Found: C, 60.32; H, 7.16; N, 19.92.

(18, Cbz-Leu-Abu-CH_2-NH-$(CH_2)_3$-2-methoxyadenine). This compound was obtained by the reaction of Cbz-Leu-Abu-CH_2Cl and 9-(3-aminopropyl)-2-methoxyadenine. The crude product was purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (8% yield). ^{1}H NMR (DMSO-d_6): 0.53-0.59 (m, 3H, Abu-CH_3), 0.79-0.85 (m, 6H, 2 x Leu-CH_3), 1.31-1.76 (m, 5H, 2 x CH_2 and CH), 1.83-2.26 (m, 6H, 3 x CH_2), 2.62 (t, 1H, NH), 3.78 (s, 3H, OCH_3), 3.95-4.06 (m, 4H, 2 x α-H and CH_2), 4.94-5.07 (m, 2H, CH_2), 7.15 (s, 2H, NH_2), 7.24-7.33 (m, 5H, Ph), 7.57 (d, 1H, NH), 7.87 (s, 1H, CH), 8.03 (d, 1H, NH). HRMS (FAB) Calcd. for $C_{28}H_{41}N_8O_5$: 569.3200. Observed *m/z* 569.3211. Anal. Calcd. for $C_{28}H_{40}N_8O_5 \cdot 0.2H_2O$: C, 58.77; H, 7.12; N, 19.58. Found: C, 58.83; H, 7.09; N, 18.75.

(19, Cbz-Leu-Abu-CH_2-NH-$(CH_2)_3$-2-chloroadenine). This compound was obtained by the reaction of Cbz-Leu-Abu-CH_2Cl and 9-(3-aminopropyl)-2-chloroadenine. The crude product was purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (13% yield). ^{1}H

NMR (DMSO-d_6): 0.54-0.59 (m, 3H, Abu-CH_3), 0.79-0.86 (m, 6H, 2 x Leu-CH_3), 1.33-1.75 (m, 5H, 2 x CH_2 and CH), 1.83-2.25 (m, 6H, 3 x CH_2), 2.60 (t, 1H, NH), 3.97-4.09 (m, 4H, 2 x α-H and CH_2), 4.95-5.07 (m, 2H, CH_2), 7.27-7.33 (m, 5H, Ph), 7.57 (d, 1H, NH), 7.70 (s, 2H, NH_2), 8.03 (s, 1H, CH), 8.10 (d, 1H, NH). HRMS (FAB) Calcd. for $C_{27}H_{37}N_8O_4Cl$: 573.2699. Observed *m/z* 573.2658. Anal. Calcd. for $C_{28}H_{40}N_8O_5 \cdot 0.15H_2O$: C, 56.32; H, 6.53; N, 19.46. Found: C, 56.33; H, 6.53; N, 18.93.

Synthetic Procedures for the Synthesis of Aza-peptidyl Ketones

N-Benzyloxycarbonylleucine hydrazide (21, Cbz-Leu-NH-NH_2). Anhydrous hydrazine (10 equiv) was added to a stirred solution of Cbz-Leu-OMe (1 eq) in MeOH and the reaction mixture was stirred vigorously for 16 h at room temperature. Excess hydrazine and solvent were removed under vacuum and the residue was washed with ether several times to give the peptidyl hydrazide as a white solid. ^{1}H NMR (DMSO-d_6): 0.81-0.88 (m, 6H, 2 x Leu-CH_3), 1.29-1.58 (m, 3H, CH_2 and CH), 3.96 (m, 1H, α-H), 4.19 (s, 2H, NH_2), 4.47 (d, 1H, NH), 4.99 (s, 2H, CH_2), 7.29-7.39 (m, 5H, Ph), 9.15 (s, 1H, NH). MS (ESI) *m/z* 280.2 [$(M + 1)^+$].

(22, Cbz-Leu-hydrazoneAbu). Cbz-Leu-NH-NH_2 (1 eq) was dissolved in dry THF and cooled down to 4 °C. Acetaldehyde (2 eq) was added to the cooled solution and the reaction mixture was continued to be stirred at 4 °C for 16 h. Solvent was removed under vacuum and the product was purified by column chromatography on silica gel using 10%MeOH/CH_2Cl_2 as the eluent; white solid, yield 92%. ^{1}H NMR (DMSO-d_6): 0.82-0.88 (m, 9H, 2 x Leu-CH_3 and Abu-CH_3), 1.32-1.64 (m, 3H, CH_2 and CH), 3.95-3.99 (m, 1H, α-H), 4.99 (s, 2H, CH_2), 7.26-7.33 (m, 6H, Ph and CH), 7.49 (d, 1H, NH), 10.85 (s, 1H, NH). MS (ESI) *m/z* 306.1 [$(M + 1)^+$].

(23, Cbz-Leu-AAbu). $NaBH_3CN$ (3 eq) was added to a solution of peptidyl hydrazone (1 eq) in dry THF. The reaction mixture was heated to 60 °C and stirred overnight after the addition of HCl. The solvent was evaporated and the residue was dissolved in EtOAc and washed with 10% $NaHCO_3$, water, saturated NaCl and dried over $MgSO_4$ and concentrated. Purification on a silica gel column with the proper eluent gave the product with 52% yield. ^{1}H NMR (DMSO-d_6): 0.81-0.94 (m, 9H, 2 x Leu-CH_3 and Abu-CH_3), 1.33-1.57 (m, 3H, CH_2 and CH), 2.62-2.66 (m, 2H, CH_2), 3.97 (m, 1H, α-H), 4.76 (s, 1H, NH), 4.99 (s, 2H, CH_2), 7.30-7.39 (m, 6H, Ph and NH), 9.41 (s, 1H, NH). MS (ESI) *m/z* 308.2 [$(M + 1)^+$].

(24, Cbz-Leu-AAbu-$(CH_2)_5$Br). To a stirred solution of the 6-bromocaproic acid (1.5 eq) in DMF at -10 °C was added HOBt (1.5 eq), the peptidyl hydrazide precursor (1 eq) and EDC (1.5 eq) was added. The mixture was allowed to react for 16 h at room temperature. The DMF was evaporated, and the residue was redissolved in EtOAc. The organic layer was washed with 2% citric acid, saturated $NaHCO_3$, saturated NaCl, dried over $MgSO_4$, and concentrated. Purification on a silica gel column with the proper eluent gave the product with 41% yield. ^{1}H NMR (DMSO-d_6): 0.92-1.03 (m, 9H, 2 x Leu-CH_3 and Abu-CH_3), 1.36-1.79 (m, 7H, 3 x CH_2 and CH), 2.18-2.12 (m, 2H, CH_2), 3.32-3.53 (m, 6H, 3 x CH_2), 4.24 (m, 1H, α-H), 5.07 (s, 2H, CH_2), 5.62 (d, 1H, NH), 7.26-7.31 (m, 5H, Ph), 8.94 (s, 1H, NH). MS (ESI) *m/z* 485.2 [$(M + 1)^+$].

General Procedure for the synthesis of Aza-peptidyl Ketones. Cbz-Leu-AAbu-$(CH_2)_5$Br (1 eq) was dissolved in DMF, anhydrous K_2CO_3 (1.5 eq)and the base (1.5 eq) were added to the solution and the reaction mixture was stirred overnight at room

temperature. The DMF was evaporated and the crude product was subjected to column chromatography to obtain the product.

(25, Cbz-Leu-AAbu-$(CH_2)_5$-adenine). This compound was obtained by reacting Cbz-Leu-AAbu-$(CH_2)_5$Br and adenine. The crude product was purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (25% yield). ^{1}H NMR (DMSO-d_6): 0.82-0.96 (m, 9H, 2 x Ile-CH_3 and Abu-CH_3), 1.16 (t, 2H, CH_2), 1.37-1.75 (m, 7H, 3 x CH_2 and CH), 2.09 (m, 2H, CH_2), 3.12 (s, 1H, CH), 3.62 (s, 1H, CH), 3.99-4.07 (m, 3H, α-H and CH_2), 4.99 (s, 2H, Cbz), 7.15-7.30 (m, 7H, Ph and NH_2), 7.63 (d, 1H, NH), 8.09 (d, 2H, 2 x Adenine-CH), 10.45 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{27}H_{39}N_8O_4$: 539.3094. Observed *m/z* 539.3118. Anal. Calcd. for $C_{27}H_{38}N_8O_4 \cdot 0.125EtOAc \cdot 0.3H_2O$: C, 59.51; H, 7.19; N, 20.19. Found: C, 59.46; H, 7.26; N, 20.19.

(26, Cbz-Leu-AAbu-$(CH_2)_5$-2-methoxyadenine). This compound was obtained by reacting Cbz-Leu-AAbu-$(CH_2)_5$Br and 2-methoxyadenine. The crude product was purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (23% yield). ^{1}H NMR (DMSO-d_6): 0.83-0.97 (m, 9H, 2 x Ile-CH_3 and Abu-CH_3), 1.13-1.16 (t, 2H, CH_2), 1.34-1.74 (m, 7H, 3 x CH_2 and CH), 2.10 (m, 2H, CH_2), 3.11 (s, 1H, CH), 3.61 (s, 1H, CH), 3.77 (s, 3H, OCH_3), 3.98-4.03 (m, 3H, α-H and CH_2), 4.99 (s, 2H, Cbz), 7.16-7.30 (m, 7H, Ph and NH_2), 7.63 (d, 1H, NH), 7.89 (s, 1H, CH), 10.46 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{28}H_{41}N_8O_5$: 569.3200. Observed *m/z* 569.3195. Anal. Calcd. for $C_{28}H_{40}N_8O_5 \cdot 0.4H_2O$: C, 58.40; H, 7.14; N, 19.46. Found: C, 58.45; H, 7.16; N, 19.33.

(27, Cbz-Leu-AAbu-$(CH_2)_5$-2-chloroadenine). This compound was obtained by reacting Cbz-Leu-AAbu-$(CH_2)_5$Br and 2-chloroadenine. The crude product was purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (18% yield). ^{1}H NMR (DMSO-d_6) 0.83-0.97 (m, 9H, 2 x Ile-CH_3 and Abu-CH_3), 1.14-1.17 (t, 2H, CH_2), 1.37-1.73 (m, 7H, 3 x CH_2 and Leu-CH), 2.10 (m, 2H, CH_2), 3.21 (s, 1H, CH), 3.62 (s, 1H, CH), 3.97-4.02 (m, 3H, α-H and CH_2), 4.95-5.03 (m, 2H, Cbz), 7.30 (m, 5H, Ph), 7.63 (d, 1H, NH), 7.71 (s, 2H, NH_2), 8.11 (s, 1H, CH), 10.46 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{27}H_{38}N_8O_4Cl$: 573.2705. Observed *m/z* 573.2710. Anal. Calcd. for $C_{27}H_{37}N_8O_4Cl \cdot 0.2H_2O$: C, 56.23; H, 6.54; N, 19.43. Found: C, 56.38; H, 6.64; N, 19.27.

(28, Cbz-Leu-AAbu-$(CH_2)_5$-N4-acetylcytosine). This compound was obtained by reacting Cbz-Leu-AAbu-$(CH_2)_5$Br and N-acetylcytosine. The crude product was purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (18% yield). ^{1}H NMR (DMSO-d_6) 0.83-0.97 (m, 9H, 2 x Ile-CH_3 and Abu-CH_3), 1.14-1.18 (t, 2H, CH_2), 1.38-1.60 (m, 7H, 3 x CH_2 and Leu-CH), 2.05-2.16 (m, 5H, CH_2 and CH_3), 3.12 (s, 1H, CH), 3.62-3.73 (m, 3H, CH_2 and CH), 4.00-4.03 (m, 1H, α-H), 5.00 (s, 2H, Cbz), 7.09 (d, 1H, NH), 7.30-7.33 (m, 5H, Ph), 7.62-7.64 (d, 1H, CH), 8.01-8.03 (d, 1H, CH), 10.46 (s, 1H, NH), 10.76 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{28}H_{41}N_6O_6$: 557.3088. Observed *m/z* 557.3065. Anal. Calcd. for $C_{28}H_{40}N_6O_6 \cdot 0.1EtOAc \cdot 0.5H_2O$: C, 59.38; H, 7.33; N, 14.63. Found: C, 59.53; H, 7.38; N, 14.53.

(29, Cbz-Leu-AAbu-$(CH_2)_5$-cytosine). This compound was obtained by reacting Cbz-Leu-AAbu-$(CH_2)_5$-N-acetylcytosine with 7N NH_3 in methanol. The crude product

was purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (82% yield). ^{1}H NMR (DMSO-d_6): 0.84-0.97 (m, 9H, 2 x Ile-CH_3 and Abu-CH_3), 1.14 (t, 2H, CH_2), 1.39-1.62 (m, 7H, 3 x CH_2 and Leu-CH), 2.09 (m, 2H, CH_2), 3.12 (s, 1H, CH), 3.53-3.61 (m, 3H, CH_2 and CH), 3.98-4.04 (m, 1H, α-H), 4.97-5.04 (m, 2H, Cbz), 5.57 (d, 1H, NH), 6.87-6.96 (s, 2H, NH_2), 7.32 (m, 5H, Ph), 7.49 (d, 1H, CH), 7.64 (d, 1H, CH), 10.47 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{26}H_{39}N_6O_5$: 515.2982. Observed *m/z* 515.3004. Anal. Calcd. for $C_{26}H_{38}N_6O_5 \cdot 0.1EtOAc \cdot 1.5H_2O$: C, 57.61; H, 7.65; N, 15.27. Found: C, 57.65; H, 7.33; N, 15.14.

N^2-(N-Benzyloxycarbonylleucyl)-N^1-ethyl-N^1-((4-morpholin-1-yl)hexanoyl)hydrazine (30, Cbz-Leu-AAbu-$(CH_2)_5$-morpholine). This compound was obtained by reacting Cbz-Leu-AAbu-$(CH_2)_5$Br and morpholine. The crude product was purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (13% yield). ^{1}H NMR ($CDCl_3$): 0.94-1.09 (m, 9H, 2 x Ile-CH_3 and Abu-CH_3), 1.45-1.80 (m, 7H, 3 x CH_2 and CH), 2.20 (m, 2H, CH_2), 2.31 (m, 4H, morpholine), 2.55-2.75 (m, 4H, 2 x CH_2), 3.52 (m, 4H, morpholine), 3.81 (s, 2H, CH_2), 4.29 (m, 1H, α-H), 5.04-5.14 (m, 2H, Cbz), 5.98 (s, 1H, NH), 7.33 (s, 5H, Ph), 9.28 (s, 1H, NH). HRMS (FAB) Calcd. for $C_{26}H_{43}N_4O_5$: 491.3234. Observed *m/z* 491.3239. Anal. Calcd. for $C_{26}H_{42}N_4O_5 \cdot 0.6CH_2Cl_2 \cdot 1H_2O$: C, 57.09; H, 8.14; N, 10.01. Found: C, 57.21; H, 8.16; N, 10.42.

N^2-(N-Benzyloxycarbonylleucyl)-N^1-ethyl-N^1-((4-methylpiperazin-1-yl)hexanoyl)hydrazine (31, Cbz-Leu-AAbu-$(CH_2)_5$-N-Me-piperazine). This compound was obtained by reacting Cbz-Leu-AAbu-$(CH_2)_5$Br and N-methyl piperazine.

The crude product was purified by column chromatography using 15% MeOH/CH_2Cl_2 as the eluent. Recrystallization with EtOAc/hexane gave a white powder (15% yield). ^1H NMR ($CDCl_3$): 0.87-1.08 (m, 9H, 2 x Ile-CH_3 and Abu-CH_3), 1.24-1.81 (m, 10H, 3 x CH_2, Leu-CH and CH_3), 2.23-2.47 (m, 6H, 3 x CH_2), 2.88 (s, 4H, piperazine), 3.53 (m, 4H, piperazine), 4.04 (t, 2H, CH_2), 4.30 (m, 1H, α-H), 5.10 (s, 2H, Cbz), 5.92 (s, 1H, NH), 7.33 (s, 5H, Ph). HRMS (FAB) Calcd. for $C_{27}H_{46}N_5O_4$: 503.3472. Observed *m/z* 503.3464. Anal. Calcd. for $C_{27}H_{45}N_5O_4 \cdot 2CH_2Cl_2 \cdot 0.5H_2O$: C, 51.03; H, 7.38; N, 10.26. Found: C, 50.91; H, 7.65; N, 10.69.

REFERENCES

1. Rawlings, N. D.; Barrett, A. J. Evolutionary families of peptidases. *Biochem. J.* **1993,** 290, 205-218.

2. Turk, B. Targeting proteases: Successes, failures and future prospects. *Nature Rev. Drug Discov.* **2006,** 5, 785-799.

3. Schechter, I; Berger, A. On size of active site in proteases. I. Papain. *Biochem. Biophys. Res. Commun.* **1967,** 27, 157-162.

4. Menard, R.; Carriere, J.; Laflamme, P.; Plouffe, C.; Khouri, H. E.; Vernet, T.; Tessier, D. C.; Thomas, D. Y.; Storer, A. C. Contribution of the glutamine 19 side chain to transition-state stabilization in the oxyanion hole of papain. *Biochemistry* **1991,** 30, 8924-8928.

5. Lecaille, F.; Kaleta, J.; Bromme, D. Human and parasitic papain-like cysteine proteases: Their role in physiology and pathology and recent developments in inhibitor design. *Chem. Rev.* **2002,** 102, 4459-4488.

6. Drenth, J.; Jansonius, J. N.; Koekoek, R.; Swen, H. M.; Wolthers, B. G. Structure of papain. *Nature* **1968,** 218, 929-932.

7. Kimmel, J. R.; Smith, E. L. The properties of papain. *Adv. Enzymol. Rel. Subj. Biochem.* **1957,** 19, 267-334.

8. Hasnain, S.; Hirama, T.; Huber, C. P.; Mason, P.; Mort, J. S. Characterization of cathepsin-B specificity by site-directed mutagenesis - Importance of Glu(245) in the S2-P2 specificity for arginine and its role in transition-state stabilization. *J. Biol. Chem.* **1993,** 268, 235-240.

9. McGrath, M. E. The lysosomal cysteine proteases. *Ann. Rev. Biophys. Biomol. Struc.* **1999,** 28, 181-204.

10. Donkor, I. O. A survey of calpain inhibitors. *Curr. Med. Chem.* **2000,** 7, 1171-1188.

11. Saez, M. E.; Ramirez-Lorca, R.; Moron, F. J.; Ruiz, A. The therapeutic potential of the calpain family: New aspects. *Drug Discov. Today* **2006,** 11, 917-923.

12. Chatterjee, S.; Iqbal, M.; Mallya, S.; Senadhi, S. E.; O'Kane, T. M.; McKenna, B. A.; Bozyczko-Coyne, D.; Kauer, J. C.; Siman, R.; Mallamo, J. P. Exploration of the importance of the P-2-P-3 -NHCO-moiety in a potent Di- or tripeptide inhibitor of calpain I: Insights into the development of nonpeptidic inhibitors of calpain I. *Bioorg. Med. Chem.* **1998,** 6, 509-522.

13. Ray, S. K. Currently evaluated calpain and caspase inhibitors for neuroprotection in experimental brain ischemia. *Curr. Med. Chem.* **2006,** 13, 3425-3440.

14. Mcgrath, M. E.; Eakin, A. E.; Engel, J. C.; Mckerrow, J. H.; Craik, C. S.; Fletterick, R. J. The crystal structure of cruzain - A therapeutic target for Chagas Disease. *J. Mol. Biol.* **1995,** 247, 251-259.

15. Gillmor, S. A.; Craik, C. S.; Fletterick, R. J. Structural determinants of specificity in the cysteine protease cruzain. *Protein Sci.* **1997,** 6, 1603-1611.

16. Huang, L.; Brinen, L. S.; Ellman, J. A. Crystal structures of reversible ketone-based inhibitors of the cysteine protease cruzain. *Bioorg. Med. Chem.* **2003,** 11, 21-29.

17. Caffrey, C. R.; Hansell, E.; Lucas, K. D.; Brinen, L. S.; Hernandez, A. A.; Cheng, J. N.; Gwaltney, S. L.; Roush, W. R.; Stierhof, Y. D.; Bogyo, M.; Steverding, D.; McKerrow, J. H. Active site mapping, biochemical properties and subcellular localization of rhodesain, the major cysteine protease of *Trypanosoma brucei rhodesiense*. *Mol. Biochem. Parasitol.* **2001,** 118, 61-73.

18. O'Brien, T. C.; Mackey, Z. B.; Fetter, R. D.; Choe, Y.; O'Donoghue, A. J.; Zhou, M.; Craik, C. S.; Caffrey, C. R.; McKerrow, J. H. A parasite cysteine protease is key to host protein degradation and iron acquisition. *J. Biol. Chem.* **2008,** 283, 28934-28943.

19. Choi, S. J.; Reddy, S. V.; Devlin, R. D.; Menaa, C.; Chung, H. Y.; Boyce, B. F.; Roodman, G. D. Identification of human asparaginyl endopeptidase (legumain) as an inhibitor of osteoclast formation and bone resorption. *J. Biol. Chem.* **1999,** 274, 27747-27753.

20. Manoury, B.; Hewitt, E. W.; Morrice, N.; Dando, P. M.; Barrett, A. J.; Watts, C. An asparaginyl endopeptidase processes a microbial antigen for class II MHC presentation. *Nature* **1998,** 396, 695-699.

21. Liu, C.; Sun, C. Z.; Huang, H. N.; Janda, K.; Edgington, T. Overexpression of legumain in tumors is significant for invasion/metastasis and a candidate enzymatic target for prodrug therapy. *Cancer Res.* **2003,** 63, 2957-2964.

22. Delcroix, M.; Sajid, M.; Caffrey, C. R.; Lim, K. C.; Dvorak, J.; Hsieh, I.; Bahgat, M.; Dissous, C.; McKerrow, J. H. A multienzyme network functions in intestinal protein digestion by a platyhelminth parasite. *J. Biol. Chem.* **2006,** 281, 39316-39329.

23. Sojka, D.; Hajdusek, O.; Dvorak, J.; Sajid, M.; Franta, Z.; Schneider, E. L.; Craik, C. S.; Vancova, M.; Buresova, V.; Bogyo, M.; Sexton, K. B.; McKerrow, J. H.; Caffrey, C. R.; Kopacek, P. IrAE - An asparaginyl endopeptidase (legumain) in the gut of the hard tick *Ixodes ricinus*. *Int. J. Parasitol.* **2007,** 37, 713-724.

24. Dixon, M. Graphical determination of equilibrium constants. *Biochem. J.* **1965,** 94, 760-762.

25. Kitz, R.; Wilson, I. B. Esters of methanesulfonic acid as irreversible inhibitors of acetylcholinesterase. *J. Biol. Chem.* **1962,** 237, 3245-3249.

26. Tian, W. X.; Tsou, C. L. Determination of the rate constant of enzyme modification by measuring the substrate reaction in the presence of the modifier. *Biochemistry* **1982,** 21, 1028-1032.

27. Steinmann, P.; Keiser, J.; Bos, R.; Tanner, M.; Utzinger, J. Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk. *Lancet Infect. Dis.* **2006,** 6, 411-425.

28. http://www.dpd.cdc.gov/dpdx/HTML/Schistosomiasis.htm.

29. Caffrey, C. R.; McKerrow, J. H.; Salter, J. P.; Sajid, M. Blood 'n' guts: an update on schistosome digestive peptidases. *Trends Parasitol.* **2004,** 20, 241-248.

30. Delcroix, M.; Sajid, M.; Caffrey, C. R.; Lim, K. C.; Dvorak, J.; Hsieh, I.; Bahgat, M.; Dissous, C.; McKerrow, J. H. A multienzyme network functions in intestinal protein digestion by a platyhelminth parasite. *J. Biol. Chem.* **2006,** 281, 39316-39329.

31. Sajid, M.; McKerrow, J. H.; Hansell, E.; Mathieu, M. A.; Lucas, K. D.; Hsieh, I.; Greenbaum, D.; Bogyo, M.; Salter, J. P.; Lim, K. C.; Franklin, C.; Kim, J. H.; Caffrey, C. R. Functional expression and characterization of *Schistosoma mansoni* cathepsin B and its trans-activation by an endogenous asparaginyl endopeptidase. *Mol. Biochem. Parasitol.* **2003,** 131, 65-75.

32. Fikrig, E.; Narasimhan, S. Borrelia burgdorferi--traveling incognito? *Microbes Infect.* **2006,** 8, 1390-1399.

33. Sojka, D.; Hajdusek, O.; Dvorak, J.; Sajid, M.; Franta, Z.; Schneider, E. L.; Craik, C. S.; Vancova, M.; Buresova, V.; Bogyo, M.; Sexton, K. B.; McKerrow, J. H.; Caffrey, C. R.; Kopacek, P. IrAE: an asparaginyl endopeptidase (legumain) in the gut of the hard tick Ixodes ricinus. *Int. J. Parasitol.* **2007,** 37, 713-724.

34. Sojka, D.; Franta, Z.; Horn, M.; Hajdusek, O.; Caffrey, C. R.; Mares, M.; Kopacek, P. Profiling of proteolytic enzymes in the gut of the tick Ixodes ricinus reveals an evolutionarily conserved network of aspartic and cysteine peptidases. *Parasit. Vectors* **2008,** 1, 7.

35. Rawlings, N. D.; Morton, F. R.; Kok, C. Y.; Kong, J.; Barrett, A. J. MEROPS: the peptidase database. *Nucleic Acids Res.* **2008,** 36, D320-D325.

36. Ishii, S. Legumain: asparaginyl endopeptidase. *Methods Enzymol.* **1994,** 244, 604-615.

37. Davis, A. H.; Nanduri, J.; Watson, D. C. Cloning and gene expression of *Schistosoma mansoni* protease. *J. Biol. Chem.* **1987,** 262, 12851-12855.

38. Abe, Y.; Shirane, K.; Yokosawa, H.; Matsushita, H.; Mitta, M.; Kato, I.; Ishii, S. Asparaginyl endopeptidase of jack bean seeds. Purification, characterization, and high utility in protein sequence analysis. *J. Biol. Chem.* **1993,** 268, 3525-3529.

39. Chen, J. M.; Stevens, R. A.; Barrett, A. J. Cloning and characterization of mouse legumain. *Mol. Biol. Cell* **1997,** 8, 2616-2616.

40. Chen, J. M.; Dando, P. M.; Rawlings, N. D.; Brown, M. A.; Young, N. E.; Stevens, R. A.; Hewitt, E.; Watts, C.; Barrett, A. J. Cloning, isolation, and characterization of mammalian legumain, an asparaginyl endopeptidase. *J. Biol. Chem.* **1997,** 272, 8090-8098.

41. Dando, P. M.; Fortunato, M.; Smith, L.; Knight, C. G.; McKendrick, J. E.; Barrett, A. J. Pig kidney legumain: an asparaginyl endopeptidase with restricted specificity. *Biochem. J.* **1999,** 339, 743-749.

42. Abdul Alim, M.; Tsuji, N.; Miyoshi, T.; Khyrul Islam, M.; Huang, X.; Motobu, M.; Fujisaki, K. Characterization of asparaginyl endopeptidase, legumain induced by blood feeding in the ixodid tick Haemaphysalis longicornis. *Insect Biochem. Mol. Biol.* **2007,** 37, 911-922.

43. Hara-Nishimura, I.; Takeuchi, Y.; Nishimura, M. Molecular characterization of a vacuolar processing enzyme related to a putative cysteine proteinase of Schistosoma mansoni. *Plant Cell* **1993,** 5, 1651-1659.

44. Choi, S. J.; Reddy, S. V.; Devlin, R. D.; Menaa, C.; Chung, H.; Boyce, B. F.; Roodman, G. D. Identification of human asparaginyl endopeptidase (legumain) as an inhibitor of osteoclast formation and bone resorption. *J. Biol. Chem.* **1999,** 274, 27747-27753.

45. Chan, C. B.; Abe, M.; Hashimoto, N.; Hao, C.; Williams, I. R.; Liu, X.; Nakao, S.; Yamamoto, A.; Li, S. Y.; Hara-Nishimura, I.; Asano, M.; Ye, K. Mice lacking asparaginyl endopeptidase develop disorders resembling hemophagocytic syndrome. *Proc. Natl. Acad. Sci. U. S. A.* **2009,** 106, 468-473.

46. Loak, K.; Li, D. N.; Manoury, B.; Billson, J.; Morton, F.; Hewitt, E.; Watts, C. Novel cell-permeable acyloxymethylketone inhibitors of asparaginyl endopeptidase. *Biol. Chem.* **2003,** 384, 1239-1246.

47. Niestroj, A. J.; Feussner, K.; Heiser, U.; Dando, P. M.; Barrett, A.; Gerhartz, B.; Demuth, H. U. Inhibition of mammalian legumain by Michael acceptors and AzaAsn-halomethylketones. *Biol. Chem.* **2002,** 383, 1205-1214.

48. Rozman-Pungercar, J.; Kopitar-Jerala, N.; Bogyo, M.; Turk, D.; Vasiljeva, O.; Stefe, I.; Vandenabeele, P.; Bromme, D.; Puizdar, V.; Fonovic, M.; Trstenjak-Prebanda, M.; Dolenc, I.; Turk, V.; Turk, B. Inhibition of papain-like cysteine proteases and legumain by caspase-specific inhibitors: when reaction mechanism is more important than specificity. *Cell Death Differ.* **2003,** 10, 881-888.

49. Gotz, M. G.; James, K. E.; Hansell, E.; Dvorak, J.; Seshaadri, A.; Sojka, D.; Kopacek, P.; McKerrow, J. H.; Caffrey, C. R.; Powers, J. C. Aza-peptidyl Michael acceptors. A new class of potent and selective inhibitors of asparaginyl endopeptidases (legumains) from evolutionarily diverse pathogens. *J. Med. Chem.* **2008,** 51, 2816-2832.

50. James, K. E.; Gotz, M. G.; Caffrey, C. R.; Hansell, E.; Carter, W.; Barrett, A. J.; McKerrow, J. H.; Powers, J. C. Aza-peptide epoxides: potent and selective inhibitors of Schistosoma mansoni and pig kidney legumains (asparaginyl endopeptidases). *Biol. Chem.* **2003,** 384, 1613-1618.

51. Mathieu, M. A.; Bogyo, M.; Caffrey, C. R.; Choe, Y.; Lee, J.; Chapman, H.; Sajid, M.; Craik, C. S.; McKerrow, J. H. Substrate specificity of schistosome versus human legumain determined by P1-P3 peptide libraries. *Mol. Biochem. Parasitol.* **2002,** 121, 99-105.

52. Engel, J. C.; Doyle, P. S.; Hsieh, I.; McKerrow, J. H. Cysteine protease inhibitors cure an experimental *Trypanosoma cruzi* infection. *J. Exp. Med.* **1998,** 188, 725-734.

53. Kurtz, A. N.; Niemann, C. Interaction of ethyl 1-acetyl-2-benzylcarbazate with alpha-chymotrypsin. *J. Am. Chem. Soc.* **1961,** 83, 1879-1881.

54. Magrath, J.; Abeles, R. H. Cysteine protease inhibition by azapeptide esters. *J. Med. Chem.* **1992,** 35, 4279-4283.

55. Ekici, O. D.; Gotz, M. G.; James, K. E.; Li, Z. Z.; Rukamp, B. J.; Asgian, J. L.; Caffrey, C. R.; Hansell, E.; Dvorak, J.; McKerrow, J. H.; Potempa, J.; Travis, J.; Mikolajczyk, J.; Salvesen, G. S.; Powers, J. C. Aza-peptide Michael acceptors: A new class of inhibitors specific for caspases and other clan CD cysteine proteases. *J. Med. Chem.* **2004,** 47, 1889-1892.

56. Asgian, J. L.; James, K. E.; Li, Z. Z.; Carter, W.; Barrett, A. J.; Mikolajczyk, J.; Salvesen, G. S.; Powers, J. C. Aza-peptide epoxides: A new class of inhibitors selective for clan CD cysteine proteases. *J. Med. Chem.* **2002,** 45, 4958-4960.

57. James, K. E.; Asgian, J. L.; Li, Z. Z.; Ekici, O. D.; Rubin, J. R.; Mikolajczyk, J.; Salvesen, G. S.; Powers, J. C. Design, synthesis, and evaluation of aza-peptide epoxides as selective and potent inhibitors of caspases-1, -3, -6, and -8. *J. Med. Chem.* **2004,** 47, 1553-1574.

58. Hogberg, T.; Strom, P.; Ebner, M.; Ramsby, S. Cyanide as an efficient and mild catalyst in the aminolysis of esters. *J. Org. Chem.* **1987,** 52, 2033-2036.

59. Nowick, J. S.; Powell, N. A.; Nguyen, T. M.; Noronha, G. An improved method for the synthesis of enantiomerically pure amino acid ester isocyanates. *J. Org. Chem.* **1992,** 57, 7364-7366.

60. Nowick, J. S.; Holmes, D. L.; Noronha, G.; Smith, E. M.; Nguyen, T. M.; Huang, S. L. Synthesis of peptide isocyanates and isothiocyanates. *J. Org. Chem.* **1996,** 61, 3929-3934.

61. Lowik, D. W. P. M.; Lowe, C. R. A stepwise synthesis of triazine-based macrocyclic scaffolds. *Tetrahedron Lett.* **2000,** 41, 1837-1840.

62. Larkins, H. L.; Hamilton, A. D. Cyclopiperazines - A new approach to chiral macrocyclic receptors. *Tetrahedron Lett.* **1986,** 27, 2721-2724.

63. Powers, J. C.; Asgian, J. L.; Ekici, O. D.; James, K. E. Irreversible inhibitors of serine, cysteine, and threonine proteases. *Chem. Rev.* **2002,** 102, 4639-750.

64. Ekici, O. D.; Li, Z. Z.; Campbell, A. J.; James, K. E.; Asgian, J. L.; Mikolajczyk, J.; Salvesen, G. S.; Ganesan, R.; Jelakovic, S.; Grutter, M. G.; Powers, J. C. Design, synthesis, and evaluation of aza-peptide Michael acceptors as selective and potent inhibitors of caspases-2, -3, -6, -7, -8, -9, and -10. *J. Med. Chem.* **2006,** 49, 5728-5749.

65. Ganesan, R.; Jelakovic, S.; Campbell, A. J.; Li, Z. Z.; Asgian, J. L.; Powers, J. C.; Grutter, M. G. Exploring the S4 and S1 prime subsite specificities in caspase-3 with aza-peptide epoxide inhibitors. *Biochemistry* **2006,** 45, 9059-9067.

66. Caffrey, C. R.; Mathieu, M. A.; Gaffney, A. M.; Salter, J. P.; Sajid, M.; Lucas, K. D.; Franklin, C.; Bogyo, M.; McKerrow, J. H. Identification of a cDNA encoding an active asparaginyl endopeptidase of *Schistosoma mansoni* and its expression in Pichia pastoris. *FEBS Lett.* **2000,** 466, 244-248.

67. Mori, K.; Iwasawa, H. Pheromone Synthesis .35. Stereoselective synthesis of optically-active forms of delta-multistriatin, the attractant for European populations of the smaller European elm bark beetle. *Tetrahedron* **1980,** 36, 87-90.

68. Methcohn, O.; Moore, C.; Taljaard, H. C. A stereocontrolled approach to electrophilic epoxides. *J. Chem. Soc., Perkin Trans. 1* **1988**, 2663-2674.

69. Meara, J. P.; Rich, D. H. Mechanistic studies on the inactivation of papain by epoxysuccinyl inhibitors. *J. Med. Chem.* **1996,** 39, 3357-3366.

70. Schaschke, N.; AssfalgMachleidt, I.; Machleidt, W.; Turk, D.; Moroder, L. E-64 analogues as inhibitors of cathepsin B. On the role of the absolute configuration of the epoxysuccinyl group. *Bioorg. Med. Chem.* **1997,** 5, 1789-1797.

71. World Health Organization, C. D. T. D. R.; 18th Program Report, U. W. T., WHO, Geneva, 1999.

72. http://www.dpd.cdc.gov/dpdx/HTML/TrypanosomiasisAmerican.htm.

73. Rangel, H. A.; Araujo, P. M. F.; Repka, D.; Costa, M. G. *Trypanosoma cruzi* - Isolation and characterization of a proteinase. *Exp. Parasitol.* **1981,** 52, 199-209.

74. Lecaille, F.; Kaleta, J.; Bromme, D. Human and parasitic papain-like cysteine proteases: Their role in physiology and pathology and recent developments in inhibitor design. *Chem. Rev.* **2002,** 102, 4459-4488.

75. Lalmanach, G.; Mayer, R.; Serveau, C.; Scharfstein, J.; Gauthier, F. Biotin-labelled peptidyl diazomethane inhibitors derived from the substrate-like sequence of cystatin: Targeting of the active site of cruzipain, the major cysteine proteinase of Trypanosoma cruzi. *Biochem. J.* **1996,** 318, 395-399.

76. Engel, J. C.; Doyle, P. S.; Hsieh, I.; McKerrow, J. H. Cysteine protease inhibitors cure an experimental Trypanosoma cruzi infection. *J. Exp. Med.* **1998,** 188, 725-734.

77. http://cdc.gov/chagas/factsheets.html.

78. http://www.dpd.cdc.gov/dpdx/HTML/TrypanosomiasisAfrican.htm.

79. Caffrey, C. R.; Hansell, E.; Lucas, K. D.; Brinen, L. S.; Alvarez Hernandez, A.; Cheng, J.; Gwaltney, S. L., 2nd; Roush, W. R.; Stierhof, Y. D.; Bogyo, M.; Steverding, D.; McKerrow, J. H. Active site mapping, biochemical properties and subcellular localization of rhodesain, the major cysteine protease of *Trypanosoma brucei rhodesiense*. *Mol. Biochem. Parasitol.* **2001,** 118, 61-73.

80. Mackey, Z. B.; O'Brien, T. C.; Greenbaum, D. C.; Blank, R. B.; McKerrow, J. H. A cathepsin B-like protease is required for host protein degradation in *Trypanosoma brucei*. *J. Biol. Chem.* **2004,** 279, 48426-48433.

81. Powers, J. C.; Asgian, J. L.; Ekici, O. D.; James, K. E. Irreversible inhibitors of serine, cysteine, and threonine proteases. *Chem. Rev.* **2002,** 102, 4639-4750.

82. Chen, Y. T.; Lira, R.; Hansell, E.; McKerrow, J. H.; Roush, W. R. Synthesis of macrocyclic trypanosomal cysteine protease inhibitors. *Bioorg. Med. Chem. Lett.* **2008,** 18, 5860-5863.

83. J Jaishankar, P.; Hartsell, E.; Zhao, D. M.; Doyle, P. S.; McKerrow, J. H.; Renslo, A. R. Potency and selectivity of P2/P3-modified inhibitors of cysteine proteases from trypanosomes. *Bioorg. Med. Chem. Lett.* **2008,** 18, 624-628.

84. Mallari, J. P.; Shelat, A.; Kosinski, A.; Caffrey, C. R.; Connelly, M.; Zhu, F.; McKerrow, J. H.; Guy, R. K. Discovery of trypanocidal thiosemicarbazone inhibitors of rhodesain and TbcatB. *Bioorg. Med. Chem. Lett.* **2008,** 18, 2883-2885.

85. Fujii, N.; Mallari, J. P.; Hansell, E. J.; Mackey, Z.; Doyle, P.; Zhou, Y. M.; Gut, J.; Rosenthal, P. J.; McKerrow, J. H.; Guy, R. K. Discovery of potent thiosemicarbazone inhibitors of rhodesain and cruzain. *Bioorg. Med. Chem. Lett.* **2005,** 15, 121-123.

86. Greenbaum, D. C.; Mackey, Z.; Hansell, E.; Doyle, P.; Gut, J.; Caffrey, C. R.; Lehrman, J.; Rosenthal, P. J.; McKerrow, J. H.; Chibale, K. Synthesis and structure-activity relationships of parasiticidal thiosemicarbazone cysteine protease inhibitors against *Plasmodium falciparum*, *Trypanosoma brucei*, and *Trypanosoma cruzi*. *J. Med. Chem.* **2004,** 47, 3212-3219.

87. Nkemngu, N. J.; Grande, R.; Hansell, E.; McKerrow, J. H.; Caffrey, C. R.; Steverding, D. Improved trypanocidal activities of cathepsin L inhibitors. *Int. J. Antimicrob. Agents* **2003,** 22, 155-159.

88. Scory, S.; Caffrey, C. R.; Stierhof, Y. D.; Ruppel, A.; Steverding, D. *Trypanosoma brucei*: Killing of bloodstream forms in vitro and in vivo by the cysteine proteinase inhibitor Z-Phe-Ala-CHN2. *Exp. Parasitol.* **1999,** 91, 327-333.

89. Gonzalez, F. V.; Izquierdo, J.; Rodriguez, S.; McKerrow, J. H.; Hansell, E. Dipeptidyl-alpha,beta-epoxyesters as potent irreversible inhibitors of the cysteine proteases cruzain and rhodesain. *Bioorg. Med. Chem. Lett.* **2007,** 17, 6697-6700.

90. Vicik, R.; Hoerr, V.; Glaser, M.; Schultheis, M.; Hansell, E.; McKerrow, J. H.; Holzgrabe, U.; Caffrey, C. R.; Ponte-Sucre, A.; Moll, H.; Stich, A.; Schirmeister, T. Aziridine-2,3-dicarboxylate inhibitors targeting the major cysteine protease of *Trypanosoma brucei* as lead trypanocidal agents. *Bioorg. Med. Chem. Lett.* **2006,** 16, 2753-2757.

91. Gotz, M. G.; Caffrey, C. R.; Hansell, E.; McKerrow, J. H.; Powers, J. C. Peptidyl allyl sulfones: A new class of inhibitors for clan CA cysteine proteases. *Bioorg. Med. Chem.* **2004,** 12, 5203-5211.

92. Asgian, J. L.; James, K. E.; Li, Z. Z.; Carter, W.; Barrett, A. J.; Mikolajczyk, J.; Salvesen, G. S.; Powers, J. C. Aza-peptide epoxides: A new class of inhibitors selective for clan CD cysteine proteases. *J. Med. Chem.* **2002,** 45, 4958-4960.

93. Ekici, O. D.; Gotz, M. G.; James, K. E.; Li, Z. Z.; Rukamp, B. J.; Asgian, J. L.; Caffrey, C. R.; Hansell, E.; Dvorak, J.; McKerrow, J. H.; Potempa, J.; Travis, J.; Mikolajczyk, J.; Salvesen, G. S.; Powers, J. C. Aza-peptide Michael acceptors: A new class of inhibitors specific for caspases and other clan CD cysteine proteases. *J. Med. Chem.* **2004,** 47, 1889-1892.

94. Li, Z. Z.; OrtegaVilain, A. C.; Patil, G. S.; Chu, D. L.; Foreman, J. E.; Eveleth, D. D.; Powers, J. C. Novel peptidyl alpha-keto amide inhibitors of calpains and other cysteine proteases. *J. Med. Chem.* **1996,** 39, 4089-4098.

95. Li, Z. Z.; Patil, G. S.; Golubski, Z. E.; Hori, H.; Tehrani, K.; Foreman, J. E.; Eveleth, D. D.; Bartus, R. T.; Powers, J. C. Peptide alpha-keto ester, alpha-keto amide, and alpha-keto acid inhibitors of calpains and other cysteine proteases. *J. Med. Chem.* **1993,** 36, 3472-3480.

96. Palmer, J. T.; Rasnick, D.; Klaus, J. L.; Bromme, D. Vinyl sulfones as mechanism-based cysteine protease inhibitors. *J. Med. Chem.* **1995,** 38, 3193-3196.

97. Renslo, A. R.; McKerrow, J. H. Drug discovery and development for neglected parasitic diseases. *Nature Chem. Biol.* **2006,** 2, 701-710.

98. Donkor, I. O.; Han, J.; Zheng, X. Z. Design, synthesis, molecular modeling studies, and calpain inhibitory activity of novel alpha-ketoamides incorporating polar residues at the P-1'-position. *J. Med. Chem.* **2004,** 47, 72-79.

99. Nowick, J. S.; Holmes, D. L.; Noronha, G.; Smith, E. M.; Nguyen, T. M.; Huang, S. L. Synthesis of peptide isocyanates and isothiocyanates. *J. Org. Chem.* **1996,** 61, 3929-3934.

100. Mallari, J. P.; Shelat, A. A.; Obrien, T.; Caffrey, C. R.; Kosinski, A.; Connelly, M.; Harbut, M.; Greenbaum, D.; McKerrow, J. H.; Guy, R. K. Development of potent purine-derived nitrile inhibitors of the trypanosomal protease TbcatB. *J. Med. Chem.* **2008,** 51, 545-552.

101. Cao, G.; Xing, J.; Xiao, X.; Liou, A. K.; Gao, Y.; Yin, X. M.; Clark, R. S.; Graham, S. H.; Chen, J. Critical role of calpain I in mitochondrial release of apoptosis-inducing factor in ischemic neuronal injury. *J. Neurosci.* **2007,** 27, 9278-9293.

102. Stys, P. K.; Jiang, Q. Calpain-dependent neurofilament breakdown in anoxic and ischemic rat central axons. *Neurosci. Lett.* **2002,** 328, 150-154.

103. Liu, M. C.; Akle, V.; Zheng, W.; Kitlen, J.; O'Steen, B.; Larner, S. F.; Dave, J. R.; Tortella, F. C.; Hayes, R. L.; Wang, K. K. Extensive degradation of myelin basic protein isoforms by calpain following traumatic brain injury. *J. Neurochem.* **2006,** 98, 700-712.

104. Kampfl, A.; Posmantur, R.; Nixon, R.; Grynspan, F.; Zhao, X.; Liu, S. J.; Newcomb, J. K.; Clifton, G. L.; Hayes, R. L. mu-calpain activation and calpain-mediated cytoskeletal proteolysis following traumatic brain injury. *J. Neurochem.* **1996,** 67, 1575-1583.

105. Shiba, E.; Kim, S.; Fujitani, M.; Kambayashi, J. I.; Kawamura, I.; Tsujimoto, S.; Shimomura, K.; Tanji, Y.; Taguchi, T.; Kimoto, Y.; Izukura, M.; Takai, S. I. Possible involvement of calpain in the growth of estrogen receptor positive breast cancer cells. *Anticancer Res.* **1996,** 16, 773-777.

106. Mamoune, A.; Luo, J. H.; Lauffenburger, D. A.; Wells, A. Calpain-2 as a target for limiting prostate cancer invasion. *Cancer Res.* **2003,** 63, 4632-4640.

107. Yang, H.; Murthy, S.; Sarkar, F. H.; Sheng, S.; Reddy, G. P.; Dou, Q. P. Calpain-mediated androgen receptor breakdown in apoptotic prostate cancer cells. *J. Cell Physiol.* **2008,** 217, 569-576.

108. Kumamoto, T.; Ueyama, H.; Watanabe, S.; Yoshioka, K.; Miike, T.; Goll, D. E.; Ando, M.; Tsuda, T. Immunohistochemical study of calpain and its endogenous

inhibitor in the skeletal muscle of muscular dystrophy. *Acta Neuropathol.* **1995,** 89, 399-403.

109. Shanmuga Sundaram, J.; Mohana Rao, V.; Meena, A. K.; Anandaraj, M. P. Altered expression, intracellular distribution and activity of lymphocyte calpain II in Duchenne muscular dystrophy. *Clin. Chim. Acta* **2006,** 373, 82-7.

110. Tang, Y. J.; Liu, X. Y.; Zoltoski, R. K.; Novak, L. A.; Herrera, R. A.; Richard, I.; Kuszak, J. R.; Kumar, N. M. Age-related cataracts in alpha 3Cx46-knockout mice are dependent on a calpain 3 isoform. *Invest. Ophthalmol.Vis. Sci.* **2007,** 48, 2685-2694.

111. Sun, M.; Zhao, Y.; Gu, Y.; Xu, C. Inhibition of nNOS reduces ischemic cell death through down-regulating calpain and caspase-3 after experimental stroke. *Neurochem. Int.* **2008**.

112. Di Rosa, G.; Odrijin, T.; Nixon, R. A.; Arancio, O. Calpain inhibitors: A treatment for Alzheimer's disease. *J. Mol. Neurosci.* **2002,** 19, 135-41.

113. Tsuji, T.; Shimohama, S.; Kimura, J.; Shimizu, K. m-Calpain (calcium-activated neutral proteinase) in Alzheimer's disease brains. *Neurosci. Lett.* **1998,** 248, 109-112.

114. Gafni, J.; Ellerby, L. M. Calpain activation in Huntington's disease. *J. Neurosci.* **2002,** 22, 4842-4849.

115. Alvira, D.; Ferrer, I.; Gutierrez-Cuesta, J.; Garcia-Castro, B.; Pallas, M.; Camins, A. Activation of the calpain/cdk5/p25 pathway in the girus cinguli in Parkinson's disease. *Parkinsonism Relat. Disord.* **2008,** 14, 309-13.

116. Samantaray, S.; Ray, S. K.; Banik, N. L. Calpain as a potential therapeutic target in Parkinson's disease. *CNS Neurol. Disord. Drug Targets* **2008,** 7, 305-312.

117. Das, A.; Guyton, M. K.; Butler, J. T.; Ray, S. K.; Banik, N. L. Activation of calpain and caspase pathways in demyelination and neurodegeneration in animal model of multiple sclerosis. *CNS Neurol. Disord. Drug Targets* **2008,** 7, 313-320.

118. Shields, D. C.; Schaecher, K. E.; Saido, T. C.; Banik, N. L. A putative mechanism of demyelination in multiple sclerosis by a proteolytic enzyme, calpain. *Proc. Natl. Acad. Sci. U. S. A.* **1999,** 96, 11486-11491.

119. Fukiage, C.; Azuma, M.; Nakamura, Y.; Tamada, Y.; Nakamura, M.; Shearer, T. R. SJA6017, a newly synthesized peptide aldehyde inhibitor of calpain: amelioration of cataract in cultured rat lenses. *Biochim. Biophys. Acta* **1997,** 1361, 304-312.

120. Iqbal, M.; Messina, P. A.; Freed, B.; Das, M.; Chatterjee, S.; Tripathy, R.; Tao, M.; Josef, K. A.; Dembofsky, B.; Dunn, D.; Griffith, E.; Siman, R.; Senadhi, S. E.; Biazzo, W.; BozyczkoCoyne, D.; Meyer, S. L.; Ator, M. A.; Bihovsky, R. Subsite requirements for peptide aldehyde inhibitors of human calpain I. *Bioorg. Med. Chem. Lett.* **1997,** 7, 539-544.

121. Donkor, I. O.; Korukonda, R.; Huang, T. L.; LeCour, L. Peptidyl aldehyde inhibitors of calpain incorporating P-2-proline mimetics. *Bioorg. Med. Chem. Lett.* **2003,** 13, 783-784.

122. Angelastro, M. R.; Mehdi, S.; Burkhart, J. P.; Peet, N. P.; Bey, P. Alpha-diketone and alpha-keto ester derivatives of N-protected amino-acids and peptides as novel inhibitors of cysteine and serine proteinases. *J. Med. Chem.* **1990,** 33, 11-13.

123. Tao, M.; Bihovsky, R.; Wells, G.; Mallamo, J. Novel peptidyl phosphorus inhibitors of human calpain I. *J. Am. Chem. Soc.* **1998,** 216, 231.

124. Tao, M.; Bihovsky, R.; Wells, G. J.; Mallamo, J. P. Novel peptidyl phosphorus derivatives as inhibitors of human calpain I. *J. Med. Chem.* **1998,** 41, 3912-3916.

125. Hanada, K.; Tamai, M.; Yamagishi, M.; Ohmura, S.; Sawada, J.; Tanaka, I. Isolation and characterization of E-64, a new thiol protease inhibitor. *Agricul. Biol. Chem.* **1978,** 42, 523-528.

126. Hanada, K.; Tamai, M.; Ohmura, S.; Sawada, J.; Seki, T.; Tanaka, I. Structure and Synthesis of E-64, a New Thiol Protease Inhibitor. *Agricul. Biol. Chem.* **1978,** 42, 529-536.

127. Barrett, A. J.; Kembhavi, A. A.; Brown, M. A.; Kirschke, H.; Knight, C. G.; Tamai, M.; Hanada, K. L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) and tts analogs as inhibitors of cysteine proteinases including cathepsins B, H and L. *Biochem. J.* **1982,** 201, 189-198.

128. Harris, A. L.; Gregory, J. S.; Maycock, A. L.; Graybill, T. L.; Osifo, I. K.; Schmidt, S. J.; Dolle, R. E. Characterization of a continuous fluorogenic assay for calpain-I - Kinetic evaluation of peptide aldehydes, halomethyl ketones and (acyloxy)methyl ketones as inhibitors of the enzyme. *Bioorg. Med. Chem. Lett.* **1995,** 5, 393-398.

129. Sasaki, T.; Kikuchi, T.; Fukui, I.; Murachi, T. Inactivation of calpain-I and calpain-II by specificity-oriented tripeptidyl chloromethyl ketones. *J. Biochem.* **1986,** 99, 173-179.

130. Higuchi, M.; Iwata, N.; Saido, T. C. Understanding molecular mechanisms of proteolysis in Alzheimer's disease: progress toward therapeutic interventions. *Biochim. Biophys. Acta* **2005,** 1751, 60-67.

131. Saatman, K. E.; Murai, H.; Bartus, R. T.; Smith, D. H.; Hayward, N. J.; Perri, B. R.; McIntosh, T. K. Calpain inhibitor AK295 attenuates motor and cognitive deficits following experimental brain injury in the rat. *Proc. Natl. Acad. Sci. U. S. A.* **1996,** 93, 3428-3433.

132. Araujo Couto, L.; Sampaio Narciso, M.; Hokoc, J. N.; Blanco Martinez, A. M. Calpain inhibitor 2 prevents axonal degeneration of opossum optic nerve fibers. *J. Neurosci. Res.* **2004,** 77, 410-419.

133. Ray, S. K.; Hogan, E. L.; Banik, N. L. Calpain in the pathophysiology of spinal cord injury: neuroprotection with calpain inhibitors. *Brain Res. Rev.* **2003,** 42, 169-185.

134. Wang, M. S.; Davis, A. A.; Culver, D. G.; Wang, Q.; Powers, J. C.; Glass, J. D. Calpain inhibition protects against Taxol-induced sensory neuropathy. *Brain* **2004,** 127, 671-9.

135. Ramamoorthy, S.; Leibach, F. H.; Mahesh, V. B.; Han, H.; Yang-Feng, T.; Blakely, R. D.; Ganapathy, V. Functional characterization and chromosomal

localization of a cloned taurine transporter from human placenta. *Biochem. J.* **1994,** 300, 893-900.

136. Weyermann, P.; Herzner, H.; Lescop, C.; Siendt, H.; Bolliger, R.; Hennebohle, M.; Rummey, C.; Briguet, A.; Courdier-Fruh, I.; Erb, M.; Foster, M.; Magyar, J. P.; von Sprecher, A.; Meier, T. Synthesis and evaluation of calpain inhibitors carrying muscle cell targeting groups. *Lett. Drug Des. Discov.* **2006,** 3, 152-158.

137. Bartus, R. T.; Hayward, N. J.; Elliott, P. J.; Sawyer, S. D.; Baker, K. L.; Dean, R. L.; Akiyama, A.; Straub, J. A.; Harbeson, S. L.; Li, Z.; Powers, J. Calpain inhibitor AK295 protects neurons from focal brain ischemia - Effects of postocclusion intraarterial administration. *Stroke* **1994,** 25, 2265-2270.

138. Wang, M. S.; Wu, Y.; Culver, D. G.; Glass, J. D. Pathogenesis of axonal degeneration: parallels between Wallerian degeneration and vincristine neuropathy. *J. Neuropathol. Exp. Neurol.* **2000,** 59, 599-606.

139. Allen, D. D.; Lockman, P. R.; Roder, K. E.; Dwoskin, L. P.; Crooks, P. A. Active transport of high-affinity choline and nicotine analogs into the central nervous system by the blood-brain barrier choline transporter. *J. Pharmacol. Exp. Ther.* **2003,** 304, 1268-74.

140. Kong, W.; Engel, K.; Wang, J. Mammalian nucleoside transporters. *Current Drug Metabolism* **2004,** 5, 63-84.

141. Gibbs, J. E.; Jayabalan, P.; Thomas, S. A. Mechanisms by which 2 ',3 '-dideoxyinosine (ddI) crosses the guinea-pig CNS barriers; relevance to HIV therapy. *J. Neurochem.* **2003,** 84, 725-734.

142. Korukonda, R.; Guan, N.; Dalton, J. T.; Liu, J.; Donkor, I. O. Synthesis, calpain inhibitory activity, and cytotoxicity of P2-substituted proline and thiaproline peptidyl aldehydes and peptidyl alpha-ketoamides. *J. Med. Chem.* **2006,** 49, 5282-5290.

143. Donkor, I. O.; Zheng, X. Z.; Miller, D. D. Synthesis and calpain inhibitory activity of alpha-ketoamides with 2,3-methanoleucine stereoisomers at the P-2 position. *Bioorg. Med. Chem. Lett.* **2000,** 10, 2497-2500.

144. Cuerrier, D.; Moldoveanu, T.; Davies, P. L. Determination of peptide substrate specificity for mu-calpain by a peptide library-based approach - The importance of promed side interactions. *J. Biol. Chem.* **2005,** 280, 40632-40641.

145. Choe, Y.; Leonetti, F.; Greenbaum, D. C.; Lecaille, F.; Bogyo, M.; Bromme, D.; Ellman, J. A.; Craik, C. S. Substrate profiling of cysteine proteases using a combinatorial peptide library identifies functionally unique specificities. *J. Biol. Chem.* **2006,** 281, 12824-12832.

146. Chatterjee, S.; Dunn, D.; Tao, M.; Wells, G.; Gu, Z. Q.; Bihovsky, R.; Ator, M. A.; Siman, R.; Mallamo, J. P. P-2-achiral, P'-extended alpha-ketoamide inhibitors of calpain I. *Bioorg. Med. Chem. Lett.* **1999,** 9, 2371-2374.

147. Donkor, I. O.; Han, J.; Zheng, X. Z. Design, synthesis, molecular modeling studies, and calpain inhibitory activity of novel alpha-ketoamides incorporating polar residues at the P-1'-position. *J. Med. Chem.* **2004,** 47, 72-79.

148. Qian, J.; Cuerrier, D.; Davies, P. L.; Li, Z. Z.; Powers, J. C.; Campbell, R. L. Cocrystal structures of primed side-extending alpha-ketoamide inhibitors reveal novel calpain-inhibitor aromatic interactions. *J. Med. Chem.* **2008,** 51, 5264-5270.

149. Li, Z. Z.; Patil, G. S.; Golubski, Z. E.; Hori, H.; Tehrani, K.; Foreman, J. E.; Eveleth, D. D.; Bartus, R. T.; Powers, J. C. Peptide alpha-keto ester, alpha-keto amide, and alpha-keto acid inhibitors of calpains and other cysteine proteases. *J. Med. Chem.* **1993**, 36, 3472-3480.

150. Li, Z. Z.; OrtegaVilain, A. C.; Patil, G. S.; Chu, D. L.; Foreman, J. E.; Eveleth, D. D.; Powers, J. C. Novel peptidyl alpha-keto amide inhibitors of calpains and other cysteine proteases. *J. Med. Chem.* **1996**, 39, 4089-4098.

151. Zhang, Q. Z.; Hua, G. X.; Bhattacharyya, P.; Slawin, A. M. Z.; Woollins, J. D. Syntheses and coordination chemistry of aminomethylphosphine derivatives of adenine. *Eur. J. Inorg. Chem.* **2003**, 2426-2437.

152. Bergmann, W.; Stempien, M. F. Contributions to the study of marine products. 43. The nucleosides of sponges. 5. The synthesis of spongosine. *J. Org. Chem.* **1957,** 22, 1575-1577.

153. Summers, W. A.; Lee, J. Y.; Burr, J. G. Synthesis of fluorescent labeled derivatives of aminopropylpyrimidines. *J. Org. Chem.* **1975,** 40, 1559-1561.

154. Graybill, T. L.; Ross, M. J.; Gauvin, B. R.; Gregory, J. S.; Harris, A. L.; Ator, M. A.; Rinker, J. M.; Dolle, R. E. Synthesis and evaluation of azapeptide-derived inhibitors of serine and cysteine proteases. *Bioorg. Med. Chem. Lett.* **1992,** 2, 1375-1380.

155. Xing, R. Y.; Hanzlik, R. P. Azapeptides as inhibitors and active site titrants for cysteine proteinases. *J. Med. Chem.* **1998,** 41, 1344-1351.